

OOCYTE MATURATION, FERTILIZATION AND POST-  
FERTILIZATION DEVELOPMENT IN TWO  
POLYCHAETE SPECIES

Gordon James Watson

A Thesis Submitted for the Degree of PhD  
at the  
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# **Oocyte Maturation, Fertilization and Post-fertilization Development in two Polychaete Species**

**by Gordon James Watson**

**Submitted for the Degree of Doctor of Philosophy in the  
University of St Andrews.**



**School of Biological & Medical Sciences**

**October 1996**



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To Mum, Dad and Ian

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## Abstract

Previous studies on *Arenicola marina* suggest that oocyte maturation is induced by a hormone, from the prostomium, acting directly on the oocyte (Meijer and Durchon, 1977). Results presented here, from studies on British populations of *Arenicola marina*, show that in this species, oocyte maturation is controlled by two hormonal steps; a prostomial maturation hormone followed by a maturation inducing substance in the coelomic fluid, the Coelomic Maturation Factor (CMF). A reliable *in vitro* assay for oocyte maturation in *A. marina* has been adopted distinguishing immature from mature oocytes enabling CMF to be investigated: CMF has a molecular mass greater than 10 kDa, is thermolabile and inactivated by trypsin suggesting a proteinaceous nature. Production of CMF was also investigated; peak production occurs at approximately 2 hours after the injection of homogenised female prostomia. Oocytes require, on average, a minimum time of 20 minutes incubation in CMF to mature and preliminary results suggest calcium may not be necessary for maturation.

Immunocytochemical techniques were used to characterise microtubule structures during oocyte maturation and post-fertilization development in *Arenicola marina*. Different regimes capable of bringing about maturation do not affect the morphology of the meiotic spindle. However, post-fertilization development is slower in oocytes matured *in vitro* when compared to mature oocytes obtained from naturally spawning females and females injected with homogenised prostomia which then spawn. Changes in microtubule structures in *A. defodiens* and *Nereis virens* during post-fertilization have also been partially characterised and these are compared and contrasted with *A. marina*. The development of a microinjection system will enable intracellular calcium and its role in maturation and fertilization within oocytes to be examined in these polychaetes.

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## List of Abbreviations

1-MeAde	1-Methyladenine
6-DMAP	6-dimethylaminopurine
ASW	Artificial sea water
BAL	British Anti-Lewisite
cAMP	cyclic adenosine monophosphate
CLSM	Confocal laser scanning microscope
CMF	Coelomic maturation factor
CSF	Cytostatic factor
DTT	DL Dithiothreitol
EGTA	Ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetra-acetic acid
ER	Endoplasmic reticulum
GLIM	Generalised linear interactive modelling
GSS	Gamete shedding substance/Gonad stimulating substance
GTP	Guanine trisphosphate
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
HDP	Hormone dependent period
HETE	8-hydroxyeicosatetraenoic acid
IP <sub>3</sub>	Inositol 1,4,5-trisphosphate
MAP	Microtubule associated protein
MD	Midzone
MIS	Meiosis-inducing substance
MPF	Maturation promoting factor
MTOC	Microtubule organising centre
PB	Polar body
pHi	Intracellular pH
PKA	Protein kinase

SA	Sperm aster
SDS-PAGE	Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SMF	Sperm maturation factor
TCA	Tricyclic anti-depressants
TFSW	Twice filtered sea water
VOS	Volatile organic substances

# **Chapter 1**

## **Introduction**

## 1.1 The Biology of *Arenicola marina*

The family Arenicolidae (Polychaeta) Johnston (1835) consists of four genera: *Arenicola* Lamarck (1801); *Branchiomaldane* Langerhans (1881); *Arenicolides* Mensil (1899) and *Abarenicola* Wells (1959). The *Arenicola* and *Abarenicola* groups, known collectively as lugworms, have an achetaous posterior region (commonly called a tail) and are termed caudate species (Wells, 1959). The other two genera are composed of species that lack a tail region and are acaudate.

There are four species of lugworm found in Britain; *Arenicola marina* (L.), *Arenicola defodiens* Cadman and Nelson-Smith (1993), *Arenicolides ecaudata* Johnston (1835), and *Arenicolides branchialis* Audouin and Milne-Edwards (1833). *Arenicola marina* is the commonest and has a wide distribution around northern Europe (Wells, 1963).

*Arenicola defodiens* has only recently been described as a separate species, although Gamble and Ashworth (1898) distinguished between two varieties of *Arenicola marina*; the "littoral" and the "laminarian". Wells (1957), however, did not consider this distinction valid and subsequently the two varieties were not distinguished in the literature. In 1993, Cadman and Nelson-Smith confirmed that the two varieties were different species with the "laminarian" variety now described as a separate species, and named *Arenicola defodiens*. According to Cadman and Nelson-Smith (1993), the differences between the two species are:

- a) genetic; the proportion of polymorphic loci and average number of alleles per locus are less in *Arenicola defodiens*.
- b) ecological; *Arenicola marina* lives higher up the shore in a U-shaped burrow of 10-30 cm depth in comparison to *A. defodiens* which is found on the lower shore, living in a J-shaped or vertical burrow, at a depth of 0.5 to 1 m.

c) morphological; *Arenicola defodiens* has a different annulation pattern at its anterior end. Its gills are pinnate with longer main stems as opposed to *A. marina*'s dendritic gills with shorter main stems. In addition the body of *A. defodiens* is usually longer and darker than *A. marina*.

*Arenicola marina* is found all around the coast of Britain in soft sediments from sandy beaches to estuarine conditions. *A. marina* is sedentary, inhabiting a characteristic U-shaped burrow, consisting of a horizontal gallery, where the worm is normally found, and vertical head and tail shafts (Wells, 1945). The tail shaft is identified by the presence of a sand cast produced by defecation which shows cyclic rhythms (Wells, 1949a, b). This cast is usually 10-20 cm away from the head hole which is characterised by a round depression in the sand (Wells, 1945).

Feeding also shows cyclic rhythms (Wells, 1949a,b). The exact nature of the diet of *Arenicola marina* is still unknown, although diatoms, organic matter and bacteria have been implicated as food sources (Hunt, 1925). The mode of feeding in *Arenicola marina* is also still debated. Deposit feeding is known to occur, with worms ingesting sand at the lower end of the head shaft (Cadée, 1976). However, Kruger (1959) suggested that *A. marina* has suspension feeding attributes as the amount of organic material in the surface of the sand was inadequate for growth by deposit feeding only. He suggested that a filtering of organic material was occurring within the sand at the head shaft. *A. marina* is also thought to actively enrich the sediment in the funnel by stimulating the growth of digestible micro-organisms with oxygenation of the sand. This is accomplished by irrigating the burrow with fresh sea water by waves of contraction passing anteriorly along the body (Hyllenberg, 1975).



*Arenicola marina* is a well-studied organism and is important for the following three main reasons:

1. *Arenicola marina* accounts for 20-30% of the benthic biomass in parts of the Dutch Wadden Sea (Beukema, 1974). It is an important food source for many species of fish; being predated heavily by plaice (de Vlas, 1979).
2. Asmus (1986) found evidence that the presence of *Arenicola marina* significantly increases the remobilisation of nutrients from sediment. Cadée (1976) also estimated that with average lugworm densities (85 per m<sup>2</sup>) in the Dutch Wadden Sea, sediment of 6-7 cm depth is reworked annually for the whole of the Dutch Wadden Sea. This would alter the depth of organic layers and, therefore, significantly effect other infaunal species.
3. The use of *Arenicola marina* as a species for bait for angling has made the lugworm commercially important and bait digging has had a profound effect on some exploited lugworm populations (Olive, 1993).

### **1.1.1 Reproductive Biology**

*Arenicola marina* is a dioecious, annual iteroparous species and under optimal conditions can reach sexual maturity towards the end of the first year of growth (de Wilde and Berghuis, 1979a). Under natural conditions though, maturity is usually reached at the end of the second year, partly due to the low winter temperatures affecting growth rates and other processes (Newell, 1948; Duncan, 1960; Cazaux, 1967).

#### **Gametogenesis**

The gonads of *Arenicola marina* remain minute in size for some time before the breeding season. There are six pairs, each situated immediately behind the coelomic funnel of each nephridium. Immature oocytes and spermatogonia are shed from the

gonads into the coelom where they continue to develop freely (Ashworth, 1904; Newell, 1948).

Most populations of *Arenicola marina* breed in the autumn (see pages 7-8 for detailed discussion). Although spring and winter populations have been reported (see Howie, 1984), the following paragraphs refer to autumn breeding populations only.

### *Oogenesis*

Ovulation takes place gradually from February until April but from then until mid-August there is a rapid increase in oocyte numbers (de Wilde and Berghuis, 1979b). Vitellogenesis begins in mid-June and by the end of August 90% of the oocytes are approaching full size of approximately 180  $\mu\text{m}$  in diameter (Rashan, 1980, Rashan and Howie, 1982). Vitellogenesis is thought to be solitary with the coelomic fluid providing the only source of oocyte nutrition during this stage. The total protein concentration within the coelomic fluid, as well as an increase in the total number of protein fractions, has been shown to increase during vitellogenesis which suggested that there was a possible heterosynthetic component to yolk production, although this has not been confirmed (Rashan, 1980, Rashan and Howie, 1982).

Although vitellogenesis may commence when worms contain oocytes of heterogenous size, the completion of oocyte growth becomes synchronised rapidly at the end of August to early September (Howie, 1984). Oocytes develop until they become arrested at the prophase of the first meiotic division at which stage they remain until immediately prior to spawning, when final maturation is initiated (Howie, 1961b).

### *Spermatogenesis*

The testes increase in size in April, May and June and coincident with this, the number of spermatogonia increases exponentially (Olive, 1972a,b). As the breeding season approaches there is a gradual accumulation in the coelomic fluid of clusters of

spermatocytes. As these develop, the clusters of spermatids can be seen to be connected in the form of a flattened plate, known as a morula (Latin for "berry"). The sperm heads face inwards and are connected to a common mass of cytoplasm, the cytophore. Once spermatogenesis is complete the sperm remain inactive within their individual morula until maturation and spawning (Bentley and Pacey, 1989).

No hormonal control of gametogenesis has been found in females. In males, however, the withdrawal of coelomic gametes from the coelom is followed by a burst of mitotic activity in the testis. Decerebration of the worm blocks this mitotic activity, but the latter could be restored if homogenised male brains were administered (Howie and McClenaghan, 1965). These authors concluded that gonadal mitosis is under the control of a substance in the male brain. This substance acts through a negative feedback mechanism inhibiting gonadal mitoses through the accumulation of gametes in the coelom.

## **Spawning**

### *Control of the Spawning Process*

Approaching the spawning season, oocytes and sperm removed directly from the coelom, although fully grown, are inactive and infertile. Only mature oocytes and activated sperm are spawned through the ciliated funnels of the nephromixia (Howie, 1961b,c, 1962). Howie (1961a,b,c) investigating the spawning process in both sexes found that only spawned gametes are fertile. He concluded that spawning of gametes was a passive process that occurred entirely as a result of gamete maturation.

In contrast to Howie (1961a,c), recent work has shown that in males spawning is an active process. The spawning process consists of periodic rhythmic muscular contractions resulting in the ejaculation of sperm through the six pairs of nephridia and lasting for about one hour (Pacey and Bentley, 1992a). Pacey and Bentley (1992a) also concluded that the Sperm Maturation Factor (SMF), putatively identified as 8,11,14 eicosatrienoic acid, acts as a true spawning hormone in addition

to its role in activating spermatozoa (see section 7.1.1 for detailed discussion). Non-gravid individuals injected with 8,11,14 eicosatrienoic acid behave in a way similar to spawning individuals (M. G. Bentley, unpublished observations).

In females the control of spawning is less understood. No obvious muscular contractions are involved and the process may take up to 24 hours (but usually around 5 hours) after injection of brain homogenate (Pacey and Bentley, 1992a). Hence, it appears that the hormone(s) controlling oocyte maturation may not have a role in inducing active muscular contractions and spawning behaviour as observed in males.

### *Spawning in the Field*

Spawning in males results in the deposition of sperm puddles on the surface of the beach at low tide (Newell, 1948; Howie, 1959; Duncan, 1960; Bentley and Pacey, 1992). In contrast, females release oocytes into their burrows which remain in the horizontal shaft (Farke and Berghuis, 1979), although the exact timing of release is unknown. As the tide returns the sperm is activated by dilution and is then washed into the female burrows where fertilization takes place (Williams *et al.*, 1996). Recently, behavioural assays with ripe females have shown that volatile organic substances (VOSs), present in the coelomic fluid of ripe males, induce burrow irrigation in the females. This would increase fertilization success by transporting spermatozoa into the burrow and therefore minimise any dilution effects (Hardege *et al.*, 1996).

### *Variation in the Timing of Spawning*

*Arenicola marina* exhibits an annual reproductive cycle generally with a single spawning period. Work by Pirlot (1933), Newell (1948) and Duncan, (1960) suggested breeding on European coasts is restricted to the autumn. However, breeding has been observed in the spring and autumn at Fairlie Sands on the west coast of Scotland (Howie, 1959) and has also been observed in early winter on the south east coast of Scotland at Dunbar (pers. obser.).

Early reports on the breeding at St Andrews were conflicting. Cunningham and Ramage (1888) observed spawning in August and September. However, Kyle (1896) stated that worms from St Andrews Bay spawn from January to September but with no spawning during April, May and early June. Subsequent observations by Howie (1959) suggested breeding occurs epidemically over 1-3 weeks in mid October, while Bentley and Pacey (1992) found that breeding occurred over two days in mid-October coincident with spring tides at east Sands, St Andrews. Spawning of this population has since been shown to be over a discrete period of 1-3 days and has been as late as early November although still coincident with spring tides (Williams *et al.*, 1996). In comparison, a population in the Eden Estuary (5 km north of East Sands), spawns over 3-4 weeks in autumn (Auckland, 1993) with small areas of the population spawning at certain times (pers. obser.). The breeding of *Arenicola marina* both in its time of year and in its extent (whether epidemic or not) is highly variable at different sites around the British coast and coasts of Europe. No direct link to a particular environmental cue has so far been established although Pirlot (1933) and Duncan (1960) noted a peak of spawning correlated to the tidal cycle and Howie (1959) observed a correlation between a drop in air temperature and spawning at St Andrews. Nevertheless, worms spawn in the laboratory irrespective of the day length, temperature, or presence of a tidal influence even when kept in isolation (Howie, 1984).

#### *Pheromonal Interactions*

In *Arenicola marina*, a number of volatile organic substances (VOSs) and non-volatile substances have been extracted from coelomic fluid of gravid males and females. The addition of oocyte-conditioned sea water to males induces significant spawning as do extracts of non-volatile substances from female coelomic fluid. Male spawning water also induces females to spawn. In both these cases it is clear that there are chemicals present, both VOSs and non-volatiles, that induce spawning in the opposite sex (Hardege *et al.*, 1996). These have yet to be characterised, but the

presence of pheromones to coordinate spawning in males and females seems in little doubt.

### **1.1.2 Larval Biology**

Development to swimming ciliated trochophores takes 3 days at 10°C. Embryos remain in the burrows for an unknown length of time. The absence of casting (production of spiral sand casts on the sand surface) by females for 3-4 weeks after spawning suggests that females stop feeding at this time to prevent ingestion of larvae, although pumping activity continues as usual (Farke and Berghuis, 1979). After this time, feeding resumes and it is likely that larvae have left the burrows before feeding recommences (Farke and Berghuis, 1979).

The destination of these larvae has been the subject of considerable debate for some years, but work by Farke and Berghuis (1979) in the Dutch Wadden Sea have shown that early larvae (up to 3 chaetigerous segments) are able to swim and move to the surface sediment. Laboratory studies have shown that at this stage they settle, build mucus tubes and feed by leaving their tubes and crawling over the surface ingesting detritus particles, although the destination of these larvae in the field remains unknown (Farke and Berghuis, 1979).

The next major stage in the development and behaviour of the larvae occurs at around 6 mm when larvae leave their mucus tubes. They secrete a new gelatinous tube enclosing the whole body and then swim in the water column (Benham, 1893). This post-larval migration of these so called Benham stages results in settlement in adult areas or areas exclusive to juveniles that are usually higher up the shore, and is determined by sediment preference (Farke and Berghuis, 1979). Migration through the sand to the adult area may then take place at a later stage although the precise mechanism by which this is achieved is not understood.



## 1.2 The Biology of *Nereis virens*

*Nereis virens* is a member of the group Nereidae which is in general an errant group of polychaetes. Often called the King Ragworm, *N. virens* is the largest species of the nereids often reaching 1 m in length (Brafield and Chapman, 1967). Worms possess a large set of eversible jaws facilitating the ingestion of animals e.g. *Nephtys hombergii*, plants and sediment (Kay, 1972).

*Nereis virens* inhabits muddy sand of the littoral and sub littoral zones of both marine and estuarine habitats. This species is found throughout boreal and temperate regions of the northern hemisphere and in Britain, populations are widespread but infrequent (Bass and Brafield, 1972). *N. virens* from some populations in Maine (USA) have been seen swimming at the surface, yet none of these worms contained gametes or any characteristic spawning or pre-spawning modifications. These "migrations" occurred during January, February and March at night on ebb tides. Individual worms were seen swimming seaward within a 20 m transect with up to 83 worms per minute counted. These movements are thought to be unrelated to reproductive behaviour but may be part of a normal behaviour pattern although the reasons for this behaviour are unknown (Dean, 1978).

The study of *Nereis virens* is important because, like *Arenicola marina*, *N. virens* is a highly prized bait species for sea angling. Natural populations are under increasing pressure from bait diggers such that the market for this species can now support commercial culture of *N. virens* in the UK.

## 1.2.1 Reproductive Biology

### Gametogenesis

#### *Oogenesis*

The site of oogonial proliferation in Nereidae is unknown due to the lack of clearly differentiated ovaries (Dhainaut, 1984). Sexual maturity is generally reached at 2-3 years (Brafield and Chapman, 1967). Under optimal conditions, however, such as in farmed animals, juveniles can reach full maturity in 6 months (Rees, pers. comm.). Brafield and Chapman (1967) found that a population at Southend, Essex (UK), had small oocytes present throughout the year and oocytes take slightly over a year to develop fully with rapid growth from September to December. Oocytes undergo vitellogenesis as solitary cells (Raven, 1961) reaching a maximum size of between 170  $\mu\text{m}$  and 180  $\mu\text{m}$  in diameter immediately prior to the spawning season. Yolk formation has been found to be of both autosynthetic and heterosynthetic origin (Fischer and Dhainaut, 1985). The Golgi apparatus is primarily responsible for autosynthetic production of yolk proteins in young oocytes. As oocytes of Nereidae become older, the Golgi apparatus becomes increasingly involved with the synthesis of mucopolysaccharides that form the prominent cortical alveoli in the peripheral cytoplasm of mature oocytes (Olive, 1983).

Heterosynthetic yolk production has been investigated using auto radiography techniques. In 1979, Fischer found a vitellin-like antigen in the body fluids of *Nereis virens* and in 1985, Fischer and Dhainaut described the uptake of radioactivity labelled *Nereis* vitellin by oocytes. The only source of vitellogenin in females has been shown to be a type of coelomocyte (eleocyte) (Fischer and Rabien, 1986; Baert and Slomianny, 1987). Transport of vitellogenin into the oocytes is thought to occur by receptor-mediated endocytosis as a vitellogenin receptor has been shown to be



present that has elements in common with other species known to have vitellogenin receptors (Hafer *et al.*, 1992).

### *Spermatogenesis*

Bass and Brafield (1972) found that the spermatozoon of *Nereis virens* was of the primitive type according to terminology used in comparative sperm morphology (Franzen, 1956). Samples of coelomic fluid taken throughout spermatogenesis by Brafield and Chapman (1967) found that recognisable sperm plates first appeared during September and October. Sperm are joined at the heads, in tetrads, until the end of January when the sperm become separated and sporadically motile. The number of motile sperm increases until the beginning of May, by which time all the sperm are active (Bass and Brafield, 1972).

### **The Reproductive Strategy**

Most nereids exhibit a semelparous mode of life; that is they reproduce only once in a lifetime and then die. *Nereis virens* displays partial "epitoky" that is the process of sexual reproduction involving swarming behaviour and a complex metamorphosis of the adult worms. As the whole worm undergoes the "metamorphosis" this is termed epigamy as opposed to schizogamy where the posterior segments of sexually mature worms become detached as migratory gamete-bearing stolons, as occurs in Syllidae. Epitoky in *Nereis virens* involves not only morphological changes but also behavioural changes and produces the so called *heteronereis*.

### **Changes Occurring During Epitoky**

Morphological changes occur to facilitate reproduction and increase the reproductive output of the animal. Each worm shows a number of morphological modifications as it approaches the breeding season. Parapodia are modified in a number of different ways and these differences vary between the male and female

worm with only the male showing major changes. A gravid male's parapodia (the 60th parapodia is taken as a typical example) increases in size to form, functionally a single paddle. In the area of its lobes alone, it is three times the size of the comparable female structure (Bass and Brafield, 1972). In males, natatory chaetae (the shafts of which are relatively resilient and unchitinized) are also produced although not on the first 30 setigers. In comparison, females do not produce these chaetae and lose a proportion of their normal chaetae (Bass and Brafield, 1972).

The changes in parapodia are not only to facilitate swimming. The development of crenellations and swellings on parapodial cirri are also involved in sexual recognition, sexual behaviour and the release of genital products (Boilly-Marer; 1974). The eyes of both sexes do not enlarge but males do show a slight swelling around the eyes and the pygidium is modified in males by the development of an anal rosette through which the seminal fluid is expelled (Bass and Brafield, 1972).

As the spawning period approaches, colour changes occur (although variable) in both the males and females. Males become dark green although this is offset by the development of the white seminal fluid within the coelom and as spawning approaches the parapodia develop a red tinge due to increased vascularisation. In females, colour changes are more variable than the males but they turn a dark green that is enhanced by the development of the green oocytes within the coelom (Bass and Brafield, 1972).

As the development of the gametes consumes nearly all the available energy and metabolites, histolysis of the body wall occurs. In males, some internal muscles (related to the intestines) are reduced and in some muscles such as the oesophageal mucosa, total atrophy occurs leaving a large lumen in contrast to the slit-like lumen of the female (Bass and Brafield, 1972). Muscles are also modified and histogenesis occurs to allow the heteronereis form to swim and release gametes which require different muscles from the atokous *Nereis* that lives in burrows (Durchon, 1984).

## Control of Gametogenesis and Reproduction

The control of the changes described above and the rest of the nereids reproductive behaviour and gametogenesis have been extensively studied and have been shown to be under hormonal control. The mechanism of endocrine control of reproduction in nereids seems to be essentially the same throughout the group (Franke and Pfannenstiel, 1984). So, although the evidence of endocrine control has been elucidated from a variety of nereid species, the results of these experiments probably also apply to *Nereis virens*.

Control of oogenesis has been shown to be due to a single hormone secreted from the supra-oesophageal ganglion, but with complicated effects (Clark, 1965). Golding (1987) suggested that the endocrine influence may be regarded as having two aspects; an inhibitory and a trophic action.

The trophic action of the hormone on gametogenesis was demonstrated by the effects of decerebration of animals at earlier stages of development that lead to the inhibition of oocyte growth (Hauenschild, 1964; Porchet, 1970; Schroeder, 1971).

Golding (1972) also suggested that the endocrine influence has an inhibitory effect on the maturation of the worm as a whole. Implanting juvenile brains into maturing *Platynereis dumerilii* reactivated regeneration and delayed metamorphosis (Hofmann and Schiedges, 1984). An *in vitro* bioassay to measure endocrine activity of brains of *Nereis diversicolor* and *Perinereis cultrifera* demonstrated that the endocrine activity diminishes during growth in both species (Durchon and Porchet, 1971). Recent work with endocrine manipulation studies on *Nereis diversicolor* has shown that the regular transplantation of immature brains into adults inhibits spawning. The mature gametes are then resorbed and a new cohort of gametes produced with this cycle continuing indefinitely. This is reminiscent of features of iteroparous species, suggesting that in *N. diversicolor* this is a vestige of an iteroparous ancestry (Golding and Yuwono, 1994).

Evidence to date suggests the existence of a single hormone controlling all aspects of reproduction in Nereidae. It is suggested that the hormone is present in high concentrations during the juvenile period supporting somatic growth and regeneration. As the process of reproduction begins a high level of hormone is required for normal development of previtellogenic oocytes and accumulation of spermatogonia but inhibits precocious vitellogenesis and spermatocyte development. The high level of hormone falls as the worm matures. Metamorphic processes needed for epitoky also require a reduction in the level of hormone. In contrast the ability to regenerate segments decreases as the hormone level declines. As sexual development continues a reduction in the hormone level is required but only beyond a critical stage can development proceed further without the hormone (Hauenschild, 1966).

The chemical nature of this hormone has only been partially determined; an active fraction obtained by High Pressure Liquid Chromatography contains a small peptide of about 2000 daltons (Cardon *et al.*, 1980).

It was thought that the brain hormone was the only endocrine substance controlling reproduction. However, work by Durchon (1952) and Porchet (1967) on *Perinereis cultrifera* showed that oocytes have a feedback role in controlling the levels of brain hormone. Porchet *et al.* (1979) showed that injection of extracts of oocytes into young females during vitellogenesis stimulated oocyte growth. It has therefore been suggested that oocytes have a positive feedback effect on the declining levels of brain hormone. As the oocytes grow they help to inhibit hormone production which, in turn stimulates oocyte growth.

### **Environmental and Pheromonal Effects**

A number of workers (Goerke, 1984; Hardege *et al.*, 1990; Bartels-Hardege and Zeeck, 1990) have shown influences of temperature and lunar cycles on spawning and swarming of nereids. No signal has yet been found which directly links endocrine control of reproduction with the environmental signals although a number

of signals may act in a complex way. Recently, it has been found that a switch to short days (8L:16D) in June led to females maturing significantly earlier than animals maintained under ambient photoperiodic conditions and that temperature has a role in maintaining the underlying rhythm of the animals (Rees and Olive, Pers. comm.).

In many species of Nereids pheromones have been shown to be involved in reproduction. In *Platynereis dumerilii* the nuptial dance (a specific reproductive behaviour trait) is triggered by a volatile ketone (Zeeck *et al.*, 1988) with two optical isomer forms being produced, the S(+) by males acting on females and vice versa for the R(-) form (Zeeck *et al.*, 1992). Coelomic fluid of many nereids has been found to have heterospecific activity in inducing spawning behaviour. Reproductive isolation may be maintained though, by temporal differences in reproduction (Boilly-Marer and Lassalle, 1980) or even differences in the threshold concentration of two species to a chemical active to both (Zeeck *et al.*, 1990).

### **Spawning in the Field and Behavioural Changes**

The spawning period of *Nereis virens* around Britain's coast varies. Breeding of a population in the Thames estuary occurs synchronously in May (Bass and Brafield, 1972) while a population in the Eden Estuary, Fife, Scotland spawned in early April in 1996 (pers. obser.). Breeding is signified by the swarming of whole populations of worms (although male and female swarming strategies may not be the same). Breeding is not necessarily over one day, and not always correlated with spring tides (Bass and Brafield, 1972).

Observations in the field and in the laboratory by Bass and Brafield (1972) showed that males swarmed and swam in circles expelling seminal fluid. The actual style of swimming in the heteronereid is different from the normal worm and in the laboratory males can perform this heteronereid swimming for up to one hour. The stimulus for swarming is unclear, although, Goerke (1984) suggested a link between temperature and swarming. Females in contrast only undergo heteronereid movement

for a few minutes and spawn in their burrows (Bass and Brafield, 1972). Once spawning has occurred males die within a few days although females may live for up to 30 days (Hardege, pers. comm.).

### **1.2.2 Larval Biology**

Bass and Brafield (1972) have described the development of larva in the laboratory. At 13°C, development to swimming ciliated trochophore occurs within 28-30 hours. By the fourth day the larvae have reached the trochophore stage and swim continuously. Five to six day old larvae have developed to the late trochophore stage where the chaetae are clearly present and chaetigerous segments are distinct. At this time the gut has formed although the larvae are still lecithotrophic and have ceased swimming. On the sixth day, the larvae are briefly planktonic (12-15 hours in the laboratory) which may serve as a dispersal stage. This stage is followed by the nectochaete stage and by the twelfth day larvae have begun to feed on the surface of the sand. Settlement is thought to occur in the sub littoral zone, but after 3 months these juveniles migrate up the beach into the adult population, by an unknown mechanism.

## **1.3 Oocyte Maturation**

### **1.3.1 Introduction**

Wilson (1925) stated that "...maturation is the completion of meiosis involving two successive meiotic divisions producing an oocyte with half the complement of chromosomes and two polar bodies". However, this definition is not entirely satisfactory as invertebrate zoologists consider "maturation" to represent the whole process of oogenesis. The phrase "meiotic maturation" was used to refer to the meiotic processes following the release from prophase arrest (Schuetz, 1969).



The process of meiosis produces cells (gametes) with only half the complement of chromosomes. With fertilization and fusion of the maternal and paternal pronuclei, the embryo has the same full complement of chromosomes as each of its parents. In males the two meiotic reduction divisions occur rapidly in the sperm at the same time as sperm differentiation. In females, the process of reducing the chromosome content can occur over an extended period of time; entry of female germ cells into meiosis occurs during the foetal or larval stages but actual completion may not occur until fertilization of the oocyte has occurred. Meiosis, although a continuous process, can for convenience be broken down into a number of different stages:

### **The First Meiotic Division**

#### *Prophase I*

This stage itself can also be divided into a number of discrete stages:

Leptotene	Chromosomes become visible and begin to contract
Zygotene	Homologous chromosomes begin to pair
Pachytene	Chromosomes become fully paired. Nucleoli become pronounced.
Diplotene	DNA synthesis that occurs in the S-phase prior to meiosis is visible; each member of a pair of chromosomes divides longitudinally to form two chromatids. Crossovers occur between non-sister chromatids.
Diakinesis	Chromosome contraction continues.

### *Metaphase I*

Nuclear membrane and nucleoli have disappeared. Each pair of homologues takes up a position on the spindle apparatus in an equatorial plane.

### *Anaphase I*

Anaphase begins when the members of one homologous pair move to opposite poles.

### *Interkinesis*

This stage involves reformation of the nuclear membrane and chromosomes elongate and become diffuse. This is a variable stage and in some cases does not exist with cells proceeding directly to meiosis II. These stages combine to produce the first meiotic division which in females produces one secondary oocyte and the first polar body.

## **The Second Meiotic Division**

### *Prophase II*

Chromosomes contract

### *Metaphase II*

Chromosomes arrange themselves on the second spindle apparatus in the equatorial plane.

### *Anaphase II*

The chromatids of each chromosome pair are pulled apart to the opposite poles by the action of the spindle fibres.

### *Telophase II*

Nuclei reform around the chromosome poles.



These stages make up the second meiotic division which produces one ovum and two polar bodies and completes the reduction divisions to leave the oocyte with half its complement of chromosomes.

Maturation is an integral part of the processes leading up to fertilization yet the relationship in different species between the timing of maturation and fertilization can be very different. Animals can be grouped into four categories relating fertilization to the maturation process (Wilson, 1925; Dalq, 1957).

All oocytes develop through to the prophase stage of the first meiosis, characterised by the germinal vesicle, at which stage they are then arrested.

#### *Category 1*

Reinitiation of meiosis from the prophase arrest is induced by fertilization and goes through to completion. Species in this category include *Nereis virens*, the bivalve, *Spisula* sp. and some species of nematodes and echinoids.

#### *Category 2*

Meiosis is reinitiated in this group by a variety of substances but is then arrested at the metaphase I stage. Fertilization takes place at this stage and reinitiates meiosis again. Species such as *Arenicola marina*, *Chaetopterus* sp. and molluscs such as *Mytilus* sp. and *Dentalium* sp. are placed in this category.

#### *Category 3*

Meiosis is also reinitiated in this group by a variety of substances but is then arrested at the metaphase II stage. Most vertebrates and the prochordate *Amphioxus* are in this category. Fertilization occurs at the metaphase II stage and reinitiates the completion of meiosis.

*Category 4*

Meiosis is reinitiated and is allowed to continue, with no arrest, to completion after which fertilization can take place. This category includes cnidarians and echinoderms where maturation is completed before fertilization.

The classification described above is not always exclusive as some species cannot be placed in a single category. This is the case with the polychaete *Sabellaria* sp. where fertilization can occur when the oocyte is at the germinal vesicle stage before the oocyte is arrested at metaphase I (Peaucellier, 1977).

The following sections describe the control of maturational processes in a number of species and phyla with a final section that examines some common themes in oocyte maturation.

### **1.3.2 Oocyte Maturation in *Arenicola marina***

Pirlot (1933) observed that ova mature in the nephromixia during oviposition. He concluded that oocytes could not be fertilized until they had been shed. Howie (1961b) found that oocytes are not physiologically mature while in the coelom, but contradictory to Pirlot (1933), maturation begins within the coelomic cavity and that maturation precedes spawning. At the time of spawning, oocytes have reached a diameter of 180  $\mu\text{m}$  and from histological examination all oocytes undergo maturation simultaneously followed immediately by spawning (Howie, 1961b).

Only mature oocytes are accepted automatically by the ciliated funnels of the nephromixia so that maturation of the oocytes in the body cavity is the immediate cause of their shedding. The mechanism by which the nephromixia distinguish between mature and immature gametes is unknown but may be due to the change in shape (becoming biconvex) of the matured oocyte (Howie, 1961b).

Howie (1963) showed that decerebrated worms did not spawn during the normal spawning season and maturation of the oocytes had not occurred. Injection of homogenised female prostomium induced the decerebrated animals to spawn and oocytes to mature normally and be fertilizable. However, male, or mixtures of male and female prostomia and implantation of intact whole prostomia failed to induce spawning in decerebrated females. (Howie, 1963). Howie concluded from these experiments that a substance within the prostomium of the females induces maturation of the oocytes and therefore initiates spawning of the oocytes. However, subsequent work by Howie (1966) showed that both male and female prostomia were capable of inducing spawning in females, although Auckland (1993) failed to induce spawning in females with male prostomia. Pacey and Bentley (1992a) confirmed the difference between males and females. Injection of the putative spawning hormone in males, 8,11,14, eicosatrienoic acid, failed to induce spawning in females. This suggests that male prostomia contain a substance that induces females to spawn but this is different from 8,11,14, eicosatrienoic acid.

The substance from female prostomia which induces spawning, was only synthesised and secreted during the breeding season and from examination of the brains, secretory cells that may be a potential source of maturation hormone were scarce. When these cells did occur, they were concentrated in the mid brain, the dorsal, vertical and oblique fibres terminating at the ventral pericapsular membrane, and in the posterior lobes. Experiments indicated that the posterior lobes alone could cause spawning when homogenised and injected into decerebrated females but other parts of the brain such as the anterior lobes had no hormone or very little as in the case of the midbrain (Howie, 1966).

Meijer and Durchon, working in France (1977), found that if oocytes are incubated with prostomia, in concentrations from 0.1 to 0.01 prostomium per ml, 100% of the oocytes will undergo germinal vesicle breakdown (GVBD). The time for GVBD to occur is dependent on the concentration of prostomia and ranges from 0.5 to

3 hours. This indicates a direct action of the hormone upon the oocyte and not involving any intermediates. However, it has recently been shown that maturation in oocytes of *Arenicola marina* will not take place following direct incubation in prostomial homogenate (Auckland, 1993). This is in contradiction to the work of Meijer and Durchon (1977). This anomaly can be explained because Meijer and Durchon almost certainly worked on *Arenicola defodiens* from Northern France (Bentley, pers. comm.) which unlike *A. marina* does not require a second endocrine step for maturation. Although the evidence is not conclusive, for ease of understanding in all future chapters the worms worked on by Meijer (1979a,b; 1980) are referred to as *Arenicola defodiens* and the worms used in this present study are referred to as *Arenicola marina*.

In *Arenicola defodiens*, Meijer (1980) found that a number of chemicals, known to affect calcium, affect oocyte maturation. These include propranolol, oxprenolol, tetracaine, procaine and lanthanum chloride. All these chemicals induce oocyte maturation suggesting a link between maturation and the intracellular free calcium concentration. However, the ionophore A23187, which is known to increase free calcium levels (Pressman, 1973), fails to induce maturation complicating the role of calcium in the maturation process (Meijer, 1980). The ability of oocytes to mature in calcium free sea water and the fact that maturation is not affected by the blocking of calcium transport through the oocyte membrane suggests that calcium may be released from internal stores to increase the free calcium concentration within the oocyte (Meijer, 1980). Unpublished results of Meijer found that the Triton X-100 fraction of oocyte cortices releases calcium if incubated with calcium free brain extract, as detected by the injection of the calcium sensitive fluorescent protein, aequorin. He therefore suggested that the brain hormone may have a direct impact on the calcium equilibrium of the oocyte cortex and that, as in other maturation systems, calcium may play a fundamental role in the activation of maturation.

Oocyte maturation can also be induced in *Arenicola defodiens* by two disulphide reducing agents dithiothreitol (DTT) and 2,3-dimercapto-propanol (BAL, British Anti-Lewisite) and this points to the possible involvement of -SH groups during maturation (Meijer, 1980).

The chemical nature of the maturation factor from the prostomium, of both species, and the second maturation factor in *Arenicola marina* coelomic fluid, their precise site of production and nature of action still remain unknown.

### 1.3.3 Oocyte Maturation in *Nereis virens*

In oocytes of *Nereis virens*, GVBD occurs after the oocytes have been released and inseminated. Oocytes could also be induced to undergo GVBD by ultra violet radiation as well as immersion in chemicals such as isotonic sodium or potassium chloride (Heilbrunn and Wilbur, 1937). However, GVBD did not occur if the oocytes had been previously immersed in sodium citrate. As sodium citrate prevents the action of calcium, Heilbrunn and Wilbur (1937) concluded that GVBD in *N. virens* oocytes is controlled by calcium release from the cortex of the oocyte.

### 1.3.4 Oocyte Maturation in other Polychaete Species

#### *Chaetopterus pergamentaceus*

In oocytes of this species, maturation proceeds in two steps with a first block at the germinal vesicle stage and a second block at metaphase I in which state they remain until fertilized (Mead, 1897). A number of results on the maturation of oocytes mechanically released into artificial sea water, magnesium free sea water and calcium free sea water were conflicting (Allyn, 1912; Scott and Lebaron, 1950; Goldstein, 1953; Zampetti-Bosseler *et al.*, 1973). The confusion that has arisen from these results is likely to be due to spontaneous maturation occurring when handling the oocytes (Ikegami *et al.*, 1976).

The calcium agonist, tetracaine (although not procaine) also induces maturation even in the absence of calcium in the medium. This result suggests a role for calcium ions in GVBD and changes in the permeability of the oocyte's membrane. Protein synthesis may also have a role in maintaining the metaphase block as treating oocytes with cyclohexamide, which inhibits protein synthesis, removes this block (Zampetti-Bosseler *et al.*, 1973).

### *Sabellaria alveolata*

In *Sabellaria alveolata*, it was thought that oocytes matured when they came into contact with sea water (Faure-Fremiet, 1924) and were arrested at metaphase I until fertilized. Work by Wilson (1968), however, showed that because not all oocytes undergo maturation upon shedding into seawater the actual stimulus was another unknown factor. This active factor was later found to be a proteolytic enzyme (or combination of enzymes) of the digestive fluid (Peaucellier, 1977).

The reasons why earlier workers such as Faure-Fremiet, (1924) and Harris, (1935) had concluded that sea water had been the maturation trigger was due to a lack of care when removing the animals from the tubes. This subsequently resulted in wounding to the gut wall and release of the proteolytic enzyme (Peaucellier, 1977). Maturation inducing activity was found to be due to three main types of enzyme all belonging to the neutral serine protease class: a) a trypsin-like enzyme of low maturation efficiency, b) a chymotrypsin like enzyme of high activity, and c) a broad specificity enzyme resembling that of subtilisins but because it was inhibited by 1-tosylamide-2-phenylethylchloromethylketone it was closer to chymotrypsin. This latter enzyme was found to have the highest maturation inducing activity. The concentration at which these substances induce maturation varies, with as little as  $2.5 \times 10^{-11}$  M for the second protease. The 50% maturation level (concentration required for 50% of the oocytes to mature after one hour incubation) is between  $5 \times 10^{-11}$  and  $2.5 \times 10^{-10}$  M depending on the batch of oocytes tested. The time required to mature also varies with concentration; with metaphase I being reached in 25 minutes at high



concentrations, but at threshold concentrations taking up to 50 minutes (Peaucellier, 1977).

Oocytes can, however, be fertilized before GVBD has occurred and develop normally (Peaucellier, 1977). This poses the question; why are there substances that induce oocyte maturation, when fertilization and normal development can occur without the need for a maturation inducing substance? Peaucellier (1977) postulated that the enhanced stability of prophase arrested oocytes allows them to be maintained within the coelom for an extended period of time.

### *Pectinaria gouldii*

Maturation in the oocytes of this species proceeds in two steps with a first block at the germinal vesicle stage and a second block at metaphase I where they remain until fertilized (Tweedell, 1966). A substance obtained from extracts of the sub-oesophageal ganglion and cement gland complex from males and females was found to activate the admission of immature oocytes into the nephromixia where they then underwent maturation. This substance was heat labile and had a molecular weight of greater than 12 kDa (Tweedell, 1980). Oocytes artificially removed from *P. gouldii* also undergo GVBD upon washing with sea water (Hylander *et al.*, 1981). Release from metaphase I at fertilization is thought to be due to a calcium influx as exposure to ionophore A23187 induces meiosis reinitiation but not in calcium free sea water (Anstrom and Summers, 1981).

### *Pomatoceros triqueter*

Oocytes of this marine worm are arrested at the germinal vesicle stage. When artificially released, a varying percentage of the oocytes undergo GVBD but must await fertilization before polar bodies can be formed (Cragg, 1939).

*Eulalia viridis*

In this littoral phyllodocid, the control of maturation is unknown at the present time. Olive (1975) found that the germinal vesicle remains intact, at least until spawning, and it was found during artificial fertilization that GVBD occurs after fertilization.

*Nephtys hombergii*

*Nephtys hombergii* possesses a spawning hormone but oocyte maturation appears to be independent of endocrine control (Olive, 1976). The maturation of *N. hombergii* oocytes occurs after spawning; i.e. GVBD occurs after release into sea water.

### 1.3.5 Oocyte Maturation in Molluscs

The maturation of oocytes in molluscs falls into category 1 and 2 according to the classification of Wilson (1925) and Dalq (1957). In category 1, oocytes are arrested once they reach the first prophase stage of meiosis e.g. the Surf Clam, *Spisula* sp. (Allen, 1953). Oocytes of many other common species of molluscs are in the same category as *Arenicola marina*, category 2, proceeding in two steps with a first block at the germinal vesicle stage and a second block at metaphase I.

Meiosis reinitiation in oocytes of *Spisula* sp. can be triggered by fertilization (Kadam and Koide, 1989), and a number of chemicals including KCl, serotonin (5-hydroxytryptamine, 5-HT) and the divalent ionophore A23187 (Allen, 1953; Schuetz, 1975; Hirai *et al.*, 1984). However, it is the action of serotonin that has been investigated in most detail. Many species of bivalve have been shown to contain serotonin in their nervous systems (Welsh and Moorhead, 1960; Malanga *et al.*, 1972; Hiripi and Osborne, 1976; Stefano and Catapane, 1977; Smith, 1982) and serotonin has also been found to induce spawning in marine bivalves (Matsutani and Nomura, 1982) suggesting a common theme across species of molluscs.



Injection of serotonin into the gonads of *Spisula solidissima* induces spawning of sperm and oocytes. Although *in vitro* incubation of oocytes in serotonin induces GVBD, the majority of oocytes that were spawned after injection of serotonin *in vivo* did not undergo GVBD (Hirai *et al.*, 1988). These differences between the effects of serotonin *in vivo* and *in vitro* on GVBD were due to the rapid action of serotonin on the neuromuscular system involved in spawning, but insufficient time for the critical threshold of serotonin to be reached for the oocytes to undergo GVBD (Hirai *et al.*, 1988).

Confirmation that serotonin is the maturation inducer in *Spisula* sp. came with the identification of serotonin receptor binding sites on *Spisula sachalinensis* oocyte membranes (Bandivdekar *et al.* 1991). The mechanism of action of serotonin was investigated by Varaskin *et al.* (1992). They found that *in vivo* as well as serotonin, the (n-6) fatty acids, but not polyunsaturated fatty acids of the (n-3) series induce GVBD in the oocytes of *S. sachalinensis*. Varaskin *et al.* (1992) proposed that GVBD in *S. sachalinensis* is induced by serotonin which then triggers an intracellular mechanism possibly involving cyclic adenosine monophosphate (cAMP), arachidonic acid, some of its metabolites and calcium to produce Maturation Promoting Factor (MPF). Calcium has been shown to be important in the transduction of serotonin signal; serotonin was ineffective at inducing maturation in calcium free sea water and serotonin was found to induce an increase in calcium uptake (Krantic *et al.*, 1991). The action of serotonin and the concurrent increase in calcium uptake was also blocked by tricyclic antidepressants that modulate calcium uptake (Juneja *et al.*, 1993).

Serotonin can also induce the release from the first block in category 2 species. Oocytes of both the Japanese Clam, *Ruditapes philippinarum*, and the freshwater mussel, *Dreissena polymorpha*, undergo GVBD upon exposure to serotonin (Osanai and Kuraishi, 1988; Guerrier *et al.*, 1993; Abdelmajid *et al.*, 1993; Fong *et al.*, 1994). Serotonin's involvement was confirmed as the serotonin receptor agonist (8-hydroxy-dipropylaminotetralin hydrobromide [8-OH-DPAT]) also induces oocyte maturation in

*D. polymorpha* (Fong *et al.*, 1994). Gobet *et al.*, (1994) also found a single class of original serotonin receptors on the oocyte surface on *R. philippinarrum*.

In oocytes of some species, the release from prophase I is pH dependent; increasing the pH of the sea water induces release in oocytes of *Patella vulgata* *in vitro* (Guerrier *et al.*, 1986). In *Cellana nigrolineata*, increasing the pH of the sea water to 8.5 produced the optimum number of maturing oocytes (Catalan and Yamamoto, 1993). It seems likely that changes in pH may also be involved in the resumption of meiosis.

### 1.3.6 Oocyte Maturation in *Asteroidea* (Starfish)

Starfish oocytes are arrested at the end of the prophase stage of meiosis I (germinal vesicle stage) until ovulation (Meijer and Guerrier, 1984). Maturation involves GVBD and extrusion of both polar bodies after which spawning occurs and oocytes are fertilisable. Maturation is under control of a hormonal system and this system has been one of the most extensively studied systems of maturation in the invertebrates.

In 1959, Chaet and McConnaughey reported that injection of a water extract of radial nerve of *Asterias forbesi* into the coelomic cavity of ripe animals induced shedding of gametes. This active substance, found within radial nerves of starfish at spawning, was named the Gamete Shedding Substance (GSS) and was found to be a polypeptide (Chaet 1966). GSS is present in radial nerves of both sexes, and is contained within granules in supporting cells of the radial nerve (Shirai *et al.*, 1986). GSS is a thermostable peptide of 22 amino acids with a molecular weight of around 2,100 daltons (Kanatani *et al.*, 1971). Its structure has been further elucidated from *Asterias amurensis* (Shirai *et al.*, 1986): It is composed of a number of components with different isoelectric points, and its major component sequenced from the N terminal is: A-E-K-(Y)-V-(G)-M-(G)-(F)-Y-M-A-V-V-(G)-(R)- (Parentheses indicate less reliability).

The stimulus that induces the production of GSS (which is only present at spawning times) is unknown, but may be an environmental signal such as temperature change: Galstoff and Loosanoff (1939) found that a rise in the temperature of the aquarium water induces spawning in sea urchins and starfishes. GSS itself does not induce maturation of isolated oocytes directly, but acts upon the ovary to produce a second substance (Kanatani and Shirai, 1967; Schuetz and Biggers, 1967). In *Asterina pectinifera* the action of GSS is initiated by a receptor mediated activation of G-proteins, resulting in the activation of adenylate cyclase and cAMP formation which then induces the production of the second substance by the follicle cells which surround the oocyte (Mita, 1992). It is this second substance that induces maturation and so Gamete Shedding Substance was renamed as Gonad Stimulating Substance (Kanatani, 1969).

The site of production of the second hormone was found to be within the follicle cells, and that the substance itself was 1-methyladenine (1-MeAde) (Kanatani, 1969; Kanatani *et al.*, 1969; Kanatani and Shirai, 1970; Hirai *et al.*, 1973). The production of 1-MeAde is not species specific (Kanatani, 1979). 1-MeAde is newly synthesised from 1-methyladenosine by splitting this substance into 1-MeAde and ribose by the action of the enzyme 1-methyladenosine ribohydrolase located in the starfish ovary (Kanatani, 1972; Kanatani and Shirai, 1972; Schuetz 1972; Shirai and Kanatani, 1972).

1-MeAde was named as the Meiosis-Inducing Substance (MIS) and induces GVBD at concentrations above  $10^{-7}$  M. Oocytes must also be exposed to 1-MeAde for a definite period, (the Hormone Dependent Period [HDP]) varying between 10 and 20 minutes per batch of oocytes for maturation to occur. (Kanatani *et al.*, 1969). 1-MeAde also performs a number of other roles in the process of spawning. These include dissolution of the cementing substance of the follicle cells allowing the oocytes to move freely, they are then forced out by contraction of the ovarian wall and

spawned. It also acts on the nervous system inducing the starfish to take up the special brooding posture.

For oocyte maturation, the active parts of 1-MeAde have been elucidated from experiments with a number of adenine derivatives (Dorée and Guerrier, 1975; Dorée *et al.*, 1976; Kanatani, 1973; Kanatani and Shirai, 1971, 1972; Schuetz, 1971, 1972). These studies have concluded a number of things, including the polar characteristics of the N1 group affect activity and all active compounds are strong bases. The binding site with the receptor also seems to be in the N9 or N7-N9 region.

The exact site of the response to 1-MeAde is the plasma membrane of the oocyte. Morisawa and Kanatani (1978), using detergents that remove the oocyte membrane, found the response to 1-MeAde can be prevented. However, it can be reinitiated by incubating the oocytes in sea water with the detergent previously used to remove the membrane in the first place. The factor therefore seems to be reconstituted on the surface of the oocyte and has been found to be heat stable and resistant to proteolytic enzymes (Morisawa and Kanatani, 1978).

The injection of 1-MeAde into fully grown oocytes fails to induce maturation confirming that oocyte maturation is not induced directly by 1-MeAde (Kanatani and Hiramato, 1970). Injection of cytoplasm from maturing oocytes after 1-MeAde treatment does, however, induce maturation (Kishimoto and Kanatani, 1976; Kishimoto 1986). The action of 1-MeAde therefore results in the production of MPF within the cytoplasm which induces maturation. The precise mechanism by which 1-MeAde induces the production of MPF is still not fully understood. Evidence has shown that a number of different molecules and metabolic pathways may be involved or associated in MPF production in starfish.

## Calcium

The importance of calcium in triggering meiosis in starfish was highlighted as early as 1925 by Dalcq. Detailed analysis of the role and effects of calcium in the

hormonal sequence from GSS to MPF has since been undertaken. In the absence of calcium ions, the follicle cells dispersed and oocytes did not mature (Cloud and Schuetz, 1973). Calcium ions can initiate oocyte maturation but as a result of their effects on the follicle cells rather than directly on the oocyte (Cloud and Schuetz, 1973). Isolated oocytes do not undergo maturation in natural sea water but upon elevation of external calcium levels, maturation is observed in populations of *Marthasterias glacilis* oocytes suggesting a role of calcium in maturation (Guerrier *et al.*, 1978).

Maturation also leads to internal changes in calcium: Free calcium concentration in the cytoplasm increased by 0.5-1.5  $\mu\text{M}$ , 2 seconds after exposure to 1-MeAde. The increase in the concentration of internal calcium lasted for 30 seconds and was proportional to the concentration of 1-MeAde added (Guerrier *et al.*, 1978; Moreau *et al.*, 1978). These results have been repeated *in vitro*: 1-MeAde and its active analogues were found to trigger the release of free calcium ions within 0.1 seconds with the release proportional to the 1-MeAde concentration. The site of the release was found to be from the plasma membrane rich vesicles obtained from the isolated cortices of intact oocytes (Dorée *et al.*, 1978). Further work by Rosenberg and Lee (1981) using atomic absorption spectrophotometry, found an increase in the total calcium in *Patiria* sp. oocytes after treatment with 1-MeAde. Ionophore A23187, however, fails to induce maturation, although it does (as with other treatments that increase intracellular calcium levels) have an additive effect on 1-MeAde by reducing the hormone dependent period. Furthermore, calcium antagonists inhibit maturation but their action can be reversed by increasing the concentration of 1-MeAde (Nemoto, 1982).

The use of microinjected calcium sensitive dyes has enabled workers to measure changes in calcium concentration in oocytes during maturation. Earlier workers had previously shown that an increase in intracellular calcium concentration occurs at maturation as described above. However, Kikuyama and Hiramoto (1991)



found that the calcium increases were neither necessary nor sufficient for maturation. 1-MeAde induced maturation of the oocytes occurred even if the calcium chelator; ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA) was injected into the oocyte, inhibiting the calcium increase. In contrast, other studies, in the same species, showed that following 1-MeAde treatment three calcium transients could be detected. Two were found in the cytoplasm and one in the nucleus with the nuclear transient occurring 20 seconds later than the first cytoplasmic transient. 1-MeAde was found to regulate the calcium transients in the nucleus and cytoplasm, but only the increase in calcium within the nucleus was essential for maturation (Santella and Kyoizuka, 1994).

Internal stores of calcium can be induced to release calcium ions through two main calcium channel receptors (Berridge, 1993). "IP<sub>3</sub>-sensitive receptors" that are responsive to the second messenger; inositol 1,4,5-trisphosphate (IP<sub>3</sub>), and "ryanodine receptors" that are relatively insensitive to IP<sub>3</sub>. Ryanodine receptors can, however, be stimulated by other agents, including ryanodine, caffeine and the naturally occurring ring metabolite cyclic adenosine diphosphate-ribose (Rakow and Shen, 1990; Lee, 1991; Buck *et al.*, 1992; Sardet *et al.*, 1992; Buck *et al.*, 1994). Injection of IP<sub>3</sub> and ryanodine have also been shown to elicit calcium transients in prophase arrested oocytes suggesting that these channels are involved in the release of calcium although the calcium spikes produced after injection failed to induce maturation of the oocytes (Stricker *et al.*, 1994). Evidence to date suggests contradictory roles for calcium so that the exact role of calcium in starfish oocyte maturation is still unclear.

### **Disulphide Reducing Agents**

Kishimoto and Kanatani (1973) found that the disulphide reducing agents, dithiothreitol (DTT) and 2,3-dimercapto-1-propanol (BAL) induced maturation in isolated oocytes of *Asterinia pectinifera* and *Asterias amuresis* while SH-blocking agents inhibited 1-MeAde induced maturation. DTT seems to exert its action on the

oocyte membrane as microinjection of DTT into the oocyte fails to induce maturation. 1-MeAde may therefore initiate maturation through the reduction of disulphide bonds in the cortex protein.

DTT, but not 1-MeAde, has been shown to induce maturation in sea cucumbers (Stevens, 1970; Kishimoto and Kanatani, 1980; Maryuma, 1980). The reduction of disulphide bonds is therefore important in other groups such as the holothurians although the mechanisms may be different from the starfish system of 1-MeAde.

### **Fatty Acid Pathway Involvement**

Arachidonic acid (AA), eicosapentaenoic and 12- and 15-dihydroxy-eicosatetraenoic acids are 20 carbon polyunsaturated fatty acids. All induce maturation in oocytes of *Asterias rubens* and *Marthasterias glacialis* suggesting these fatty acids may have a role in maturation. However, only AA has the same maturation kinetics as 1-MeAde (Meijer *et al.*, 1984; Meijer *et al.*, 1986a).

Arachidonic acid oxidation by the lipoxygenase pathway produces the (8S) and (8R) isomers of 8-hydroxyeicosatetraenoic acid (HETE). Only the (8R)-HETE isomer induces maturation of starfish oocytes. Starfish oocytes can metabolise AA into 8-HETE and other HETEs but only (8R)-HETE is produced from plasma membrane phospholipids suggesting that (8R) HETE might play a role in the transduction of 1-MeAde at the plasma membrane level (Meijer *et al.*, 1986a).

### **Involvement of Cyclic Nucleotides (cyclic AMP [cAMP] and cyclic GMP [cGMP])**

#### *Evidence for a Fundamental Role of cAMP in Oocyte Maturation*

Exposure of oocytes of *Marthasterias glacialis* and *Evasterias troschelii* to 1-MeAde results in a drop in cyclic 3',5' adenosine monophosphate (cAMP) levels (Mazzei *et al.*, 1981; Meijer and Zarutskie, 1987). This drop in cAMP levels in

*Evasterias troschelii* is more obvious if the oocytes are pre-treated with forskolin. Forskolin, a diterpene, is known to activate the enzyme adenylate cyclase which catalyses the reaction of cAMP production from ATP and therefore increases cAMP levels. Conversely, an increase in the concentration of cAMP in an oocyte inhibits maturation (Meijer and Zarutskie, 1987). Microinjection of the catalytic subunit (C) of cAMP-dependent protein kinase inhibits maturation when performed during the hormone dependent period. This is because cAMP's effects result through the activation of protein kinases which phosphorylate proteins (Dorée *et al.*, 1981). These studies suggest that cAMP controls maturation by maintaining the prophase arrest until 1-MeAde reduces the cAMP concentration.

#### *Evidence Against the Fundamental Role of cAMP in Oocyte Maturation*

Other studies have shown that the importance of cAMP may have been exaggerated; cAMP concentrations have been shown to increase during maturation (Dorée *et al.*, 1981), in direct contradiction to other work. The microinjection of cyclic nucleotide phosphodiesterase that degrades cAMP, does not trigger maturation (Meijer and Zarutskie, 1987) as does the injection of the R subunit of type 1 cAMP-dependent protein kinase and the heat stable inhibitor (PKI) of cAMP protein kinase (Dorée *et al.*, 1981).

#### *Possible Mechanism of cAMP Action on Maturation*

The maintenance of cAMP at high levels in oocytes is thought to maintain the prophase arrest. The reduction of cAMP levels by 1-MeAde then triggers maturation through a double pathway. The inhibition of a cAMP-dependent inhibitory pathway of protein kinase leads to permissive conditions to allow protein phosphorylation to take place. Although necessary, this is not sufficient to induce maturation on its own (Meijer *et al.*, 1989b). Cyclic 3',5' guanine monophosphate (cGMP) could also be involved as well or instead of cAMP (Meijer *et al.*, 1989b). The role of these cyclic



nucleotides in starfish oocyte maturation is still not fully understood or confirmed as having a fundamental role in oocyte maturation.

### **Protein Phosphorylation**

$\alpha$ -Naphthylphosphate ( $\alpha$ -NP) is an inhibitor of phosphatases and protein phosphatase (Li, 1984) which remove the phosphate groups from substrates. Injection of these protein phosphatases into oocytes inhibits maturation (Meijer *et al.*, 1986b). However, the injection of  $\alpha$ -NP into oocytes induces maturation (Pondaven and Meijer, 1986). This induction of maturation occurs before MPF has appeared as the effects of  $\alpha$ -NP are inhibited by nicotinamide and lithium which inhibit MPF (Picard and Dorée, 1979). Pondaven and Meijer (1986) therefore concluded that an inhibition of phosphatases leads to a gradual increase in the level of phosphorylation of proteins, which then participate in the production/activation of MPF, leading ultimately to maturation. MPF has since been elucidated in starfish and other phyla as the same substance and its common role is described in section 1.3.9.

### **1.3.7 Oocyte Maturation in other Invertebrate Groups**

Although the processes of maturation have been most extensively studied in echinoderms, molluscs, and to a lesser extent the annelids, a number of other groups have also been investigated:

#### **Cnidarians**

In the hydrozoan, *Hydractinia echinata*, spawning and oocyte maturation is thought to be induced by exposure to light after a period of dark. A hypothesis to explain this is that darkness allows the accumulation of light-sensitive substances, whose breakdown initiates maturation of the oocytes and subsequent spawning (Ballard, 1942). However, no substances have been implicated in this phenomenon.

The jellyfish *Spirocodon saltatrix* also spawns soon after nightfall. Work by Ikegami *et al.* (1978) showed that isolated ovaries discharge oocytes 50-70 minutes

after a 30 minute exposure to the dark, during which a spawning inducing substance of low molecular weight is produced in the ovaries. This substance also induces maturation of the oocytes which proceed to the 2nd polar body stage where they are then arrested until fertilization (Freeman, 1987). Oocytes only require a short time period of exposure to the hormone (the first few minutes of the maturation process) in order to complete maturation. The hormone is trypsin sensitive and preliminary studies indicate it is a peptide (Freeman, 1987). Grimmelikhuijzen (1985), using antibodies, found a peptide (Arg-Phe-amide) within the nerve plexus that could be the substance responsible for inducing maturation. The actual mechanism of maturation is unknown, although, Freeman and Ridgway (1988) found that the injection of cAMP into oocytes induced maturation in several species of hydrozoans suggesting the involvement of cAMP machinery.

Oocytes of another hydrozoan, *Phialidium gregarium*, are arrested at the germinal vesicle stage and upon maturation proceed to the 2nd polar body stage at which stage they remain until fertilization (Freeman and Ridgway, 1988). Oocyte maturation can be induced by calcium ionophore A23187 and  $\text{NH}_4\text{Cl}$  treatment. Ionophore treatment induces the production of calcium transients whilst ammonia treatment induces a rise in intracellular pH ( $\text{pH}_i$ ). This suggests that the activation pathways involving calcium and changes in  $\text{pH}_i$  are different (Freeman and Ridgway, 1993).

### 1.3.8 Oocyte Maturation in Amphibians

Amphibians have been one of the most extensively studied groups of vertebrates in relation to oocyte maturation. One species in particular, the African Clawed Toad, *Xenopus laevis* has been the most studied. Fully grown *Xenopus* oocytes are arrested in prophase of meiosis I and can be induced to mature upon exposure to progesterone *in vitro*. Progesterone, which is produced by the follicle cells surrounding the oocyte, is under the control of gonadotrophic hormones from the pituitary gland.

Oocyte maturation involves GVBD and the extrusion of the first polar body at which time development is arrested at metaphase II until fertilization (Masui and Clarke, 1979). Maturation of *Xenopus* oocytes occurs after 6-10 hours of progesterone exposure *in vitro* (Baulieu and Schorderet-Slatkine, 1983). Microinjection of progesterone fails to induce maturation suggesting that progesterone acts on the surface of the oocyte (Masui and Markert, 1971; Godeau *et al.*, 1978; Ishikawa *et al.*, 1977). Baulieu *et al.*, (1975) found a progesterone receptor on the surface that was unlike the classical steroid hormone receptor. Its specificity was found to be broad and affinity weak as other steroids have also been found to initiate maturation such as testosterone and 11-deoxycorticosterone, (Schorderet-Slatkine, 1972). Blondeau and Baulieu (1984) observed that a protein fraction in the oocyte membrane showing specific binding of progesterone had a molecular weight of approximately 30 kDa.

Once progesterone has bound to the oocyte receptor the pathway it acts upon remains largely unknown. Deshpande and Koide, (1985) found that a metabolite of progesterone, 4-pregnene- $\alpha$ ,29 $\beta$ -diol-3-one was just as active as progesterone in inducing maturation in *Xenopus* oocytes *in vitro*. This evidence suggested that *in vivo*, this might be the active metabolite of maturation induction.

Evidence suggests that progesterone, or an active metabolite, may act on cAMP machinery. Progesterone was found to inhibit adenylate cyclase bound to the membrane resulting in a decrease in cAMP (Godeau *et al.*, 1981; Jordana *et al.*, 1981; Sadler and Maller, 1981; Schorderet-Slatkine *et al.*, 1982). Forskolin treated oocytes fail to undergo maturation confirming cAMP involvement, as forskolin is known to increase adenylate cyclase activity thereby increasing the cAMP concentration (Schorderet-Slatkine and Baulieu, 1982). Sadler and Maller (1981) found that this hormonal inhibition of adenylate cyclase requires the G/F subunit of adenylate cyclase and involves, at least in part, a decrease in the rate of guanine nucleotide exchange on the membrane surface.

Calcium and intracellular pH changes may also have a role in the transduction of the progesterone signal: The injection of EGTA into oocytes inhibits progesterone induced maturation (Masui and Clarke, 1979). Other investigators have shown that the concentration of intracellular calcium increases in an oocyte during maturation (Moreau *et al.*, 1980; Wasserman *et al.*, 1980). Intracellular pH changes also occur during maturation, although these changes may not be essential for maturation they may be a consequence of other processes (Lee and Steinhardt, 1981). Recently the induction of maturation with an antibody raised against the COOH-terminal decapeptide of the  $\alpha$  subunit of the G-protein;  $G_s$ , which it inhibits, suggests that progesterone may act by inhibiting the  $\alpha_s$  subunit found on the plasma membrane and yolk platelet membranes (Gallo *et al.*, 1995).

If a small amount of cytoplasm from a progesterone incubated oocyte is removed and injected into an oocyte that has not been exposed to the hormone, maturation of this oocyte occurs within 2-3 hours. This indicates that another substance, MPF is produced from within the oocyte which induces maturation (Masui and Markert, 1971; Schorderet-Slatkine and Drury, 1973). Auto-amplification of MPF within an oocyte has been shown to occur in *Xenopus laevis* oocytes. MPF activity could be serially transferred by sequential injections into oocytes as many as 5 to 10 times without any loss of activity (Reynhout and Smith, 1974; Drury and Schorderet-Slatkine, 1975). MPF appears shortly before maturation begins and attains maximum activity at GVBD (Masui and Clarke, 1979). During maturation a two-fold increase in protein synthesis has also been recorded in *Xenopus* oocytes (Wasserman *et al.*, 1982).

MPF activity is responsible for co-ordinating the biochemical events of meiosis I and II (Darr *et al.*, 1991; Kanki and Donoghue, 1991; Sagata *et al.*, 1988; Sagata *et al.*, 1989b). However, another factor termed Cytostatic Factor (CSF) has been discovered that is calcium sensitive and responsible for the arrest of the oocyte at meiosis II and for the stabilisation of MPF (Masui and Clarke, 1979; Newport and

Kirschner, 1984; Murray *et al.*, 1989; Sagata *et al.*, 1989b). The synthesis of another protein, pp39<sup>mos</sup> (the *mos* protooncogene product), has been shown to be required for the activation of MPF (Sagata *et al.*, 1989a). The *mos* product is also an active component of CSF (Sagata *et al.*, 1989b), although meiosis II and CSF arrest also require additional proteins as well as *mos* which are synthesised *de novo* (Yew *et al.*, 1992).

### 1.3.9 Comparative Aspects of Oocyte Maturation

The roles of calcium, cyclic nucleotides, intracellular pH changes, various chemicals, hormones and chemical reactions in the process of oocyte maturation have been discussed in detail within an individual group or species. Yet, only one substance has been shown to have the same action across all species and groups and is a unifying theme among oocyte maturation in all species studied. This substance is the Maturation Promoting Factor (MPF).

#### Maturation Promoting Factor (MPF)

MPF activity is responsible for coordinating the biochemical events of meiosis I and II in oocyte maturation. MPF has been confirmed as a fundamental and universal regulator of entry into M-phase in meiosis and is ubiquitous in all eukaryotes so far tested, and is also required for the transition into M-phase of mitosis (Nurse, 1990). An active part of MPF is complexed with other different proteins and different forms of this complex have been observed in yeast, frog, clam, sea urchin and starfish cells (Arion *et al.*, 1988; Draetta and Beach, 1988; Gautier *et al.*, 1988; Wittenberg and Reed, 1988; Draetta *et al.*, 1989; Labbe *et al.*, 1989; Meijer *et al.*, 1989a).

#### *The Action of MPF*

In controlling oocyte maturation the action of MPF is essentially the same in a number of groups. In *Xenopus* the appearance of MPF in cells causes an immediate increase in protein phosphorylation. The production of MPF has also been shown to



require protein synthesis as treatment of oocytes with cycloheximide (a protein synthesis inhibitor) inhibits MPF production (Wasserman and Masui, 1975). Cytoplasm containing MPF, however, can induce recipient oocytes to complete meiotic maturation even in the presence of cycloheximide (Wasserman and Masui, 1975). This suggests that progesterone induces protein synthesis to activate MPF which then triggers maturation without the requirement of protein synthesis. The proteins required for maturation are translation products of stored maternal messenger RNA (mRNA). The regulation of this process is unclear but does require poly(A) tail elongation of the mRNA (Kuge and Inoue, 1992). MPF action is also calcium independent as injection of EGTA into progesterone-stimulated oocytes after the appearance of MPF did not inhibit maturation (Masui *et al.*, 1977).

Purification has shown a 32 kDa serine/threonine protein kinase subunit and a 45 kDa phosphoprotein, are contained within active fractions of MPF (Lohka *et al.*, 1988). The 32 kDa subunit was subsequently identified as p34<sup>cdc2</sup>, a *Xenopus* homologue of the cdc2+ gene product from the yeast, *Schizosaccharomyces pombe* (Dunphy *et al.*, 1988; Gautier *et al.*, 1988). Gautier *et al.*, (1990) have demonstrated that B-type cyclins are components of highly purified MPF and cyclin is necessary to activate the latent protein kinase activity of p34<sup>cdc2</sup>. A proposed mechanism for MPF production is that cyclin is newly synthesised and joins pre-existing p34<sup>cdc2</sup> to form pre-MPF, which forms active MPF by phosphorylation and dephosphorylation of the cdc2 subunit (Gautier *et al.*, 1990). The synthesis of another protein, pp39<sup>mos</sup> (the *mos* protooncogene product), has been shown to be required for the activation of MPF (Sagata *et al.*, 1989a).

A cAMP-dependent protein kinase (PKA) has been implicated as a negative regulator of transition in *Xenopus* oocytes into meiosis. Progesterone is believed to decrease the cAMP concentration leading to a decrease in PKA activity resulting in the dephosphorylation of a presumptive maturation-inhibiting phosphoprotein activating MPF (Smith, 1989). In the mollusc, *Ruditapes philippinarum*, 3 minutes after

serotonin addition, tyrosine phosphorylation of p42<sup>mapk</sup>, a protein related to the recently cloned extracellular signal related protein kinases (ERK) occurs. In *Spisula solidissima* the phosphorylation is transient and is followed by tyrosine dephosphorylation of p34<sup>cdc2</sup>, which activates MPF simultaneously with GVBD. In *R. philippinarum* no dephosphorylation was observed which may account for the metaphase I block in *R. philippinarum* and no block in *S. solidissima* oocytes (Abdelmajid *et al.*, 1994).

Studies have shown that p34<sup>cdc2</sup> is only one member of a large family of related kinases and several of these kinases are clearly involved in cell cycle control as well as in other functions in the cell. The catalytic subunit, p34<sup>cdc2</sup>, associates with a variety of cyclins and cyclin B has also been shown to be only one member of a family of cyclins that may be involved in MPF regulation (Lew *et al.*, 1991).

## 1.4 Objectives of the Thesis

In marine invertebrate reproductive biology, oocyte maturation, fertilization and post-fertilization development have all been examined in detail, but primarily in the phylum Echinodermata. In contrast, corresponding research into these systems in protostomes has trailed behind. Of the protostome phyla, annelids, and specifically the group polychaetes have been neglected in these research areas, consequently the level of understanding of the systems in this group is behind other phyla.

The understanding of the reproductive biology of *Arenicola marina* and *Nereis virens* is important because of the commercial and ecological significance of these species. Furthermore, this research will enable comparative model systems of oocyte maturation, fertilization and post-fertilization development to be proposed. These systems could then be evaluated with a view to using them as model systems that would parallel work in other protostome groups.

The specific objectives of the study are:

1. The development of a reliable *in vitro* assay for oocyte maturation in *Arenicola marina*.
2. To use this assay in the characterisation and purification and mode of action of the second substance from the coelomic fluid that induces oocyte maturation in *Arenicola marina*.
3. The development of immunocytochemical techniques to characterise the changes in microtubule structures during oocyte maturation in *Arenicola marina* and to compare and contrast with the recently described species *Arenicola defodiens*.
4. An investigation into the signals controlling maturation and fertilization within oocytes of *Arenicola marina* and *Nereis virens*. Specifically, changes in the internal calcium concentration will be investigated using a microinjection system.
5. To characterise changes in microtubule structures during post-fertilization development in *Arenicola marina* and *Nereis virens* using immunocytochemical techniques and to compare and contrast any differences in the species.
6. A preliminary investigation into the effect of different regimes capable of bringing about maturation on development and microtubule structures in *Arenicola marina*.



## **Chapter 2**

### **Characterisation of Oocyte Maturation in *Arenicola marina***

## 2.1 Introduction

### 2.1.1 The Visualisation of Oocyte Maturation using Chromosomal Characteristics and the Development of an *In vitro* Assay for Maturation

The investigation of the hormonal control of oocyte maturation in *Arenicola marina* requires a reliable assay for maturation. Oocytes of *Arenicola* sp. undergo a number of morphological changes as they mature; a general change in shape, retraction of the microvilli, cortical granule changes and the breakdown of the germinal vesicle (GVBD) including chromosome migration (Howie, 1961a; Meijer, 1979a). The usual method for scoring the maturation of *A. marina* oocytes has been to use bright field microscopy to examine the breakdown of the germinal vesicle. This however, is not a reliable method of determining if maturation has occurred because the high levels of yolk within oocytes can lead to considerable errors in the estimation of the extent of GVBD. The use of the fluorescent compounds known as Hoechst dyes provides a much faster and more reliable means of assaying maturation.

The assay used here, relies on the use of the bisbenzimidazole compounds known as Hoechst 33342 and 33258. These dyes bind to the minor groove of DNA, preferentially to contiguous AT base pairs (Müller and Gautier, 1975; Latt and Stetten, 1976; Pjura *et al.*, 1987), and are excited at 345 nm and emit at 460 nm (Latt and Stetten, 1976). The dyes are cell permeant, with Hoechst 33342 being slightly more cell permeant than 33258 (Arndt-Jovin and Jovin, 1977). Both are relatively non toxic (Arndt-Jovin and Jovin, 1977). Upon binding to the chromosomes the dyes fluoresce bright blue in comparison with a pale blue of the oocyte cytoplasm. This permits their relative positions to be easily identified so that mature and immature eggs can be distinguished by the differences in chromosome position and form. The permeant nature of the dyes and low toxicity allows living as well as fixed oocytes to be stained with the dyes.

The characterisation of maturation by the examination of chromosome characteristics allows numbers of mature versus immature eggs to be counted quickly and accurately. The assay permits the assessment *in vitro* of the presence of maturation inducing activity of coelomic fluid, and the ability of other test solutions to induce oocyte maturation to be evaluated.

### **2.1.2 Visualisation of Oocyte Maturation using Microtubule Characteristics**

The labelling of microtubule structures with fluorescent antibodies in conjunction with the use of Hoechst dyes allows the visualisation of changes in microtubular structures during maturation. Specifically, it can enable the appearance of the meiotic spindle and a time series of events occurring during maturation to be recorded.

The internal framework of the eukaryotic cell, termed the cytoskeleton, is composed of three types of proteinaceous structure: actin filaments, intermediate filaments and microtubules. These elements contribute to the shape of the cell, movement of cell organelles and structures, and cell locomotion (Bray, 1992).

#### **The Composition of Microtubules**

Microtubules are made of the globular, slightly acidic protein, tubulin. It has a molecular weight of 110 kDa with a subunit molecular weight of 55 kDa, confirming that tubulin is a dimer. Each dimer was found to consist of one  $\alpha$  and one  $\beta$  tubulin (the two isoforms of tubulin). Each heterodimer of tubulin in solution is known to contain two molecules of guanine trisphosphate (GTP), one of which is hydrolysed during the course of polymerisation (Bray, 1992).

#### **The Structure of Microtubules**

The microtubule wall is built from linear heterodimers arranged in a regular fashion, with the long axis of the dimer parallel to that of the cylinder (Lackie, 1985).

Microtubules are polar structures due to the asymmetric structure of each tubulin molecule. Thirteen linear protofilaments, each of 5 nm diameter, are formed along the axis of the microtubule with a total diameter of 25 nm with a central lumen of 7 nm. There are other proteins involved in the structure and these are termed microtubule associated proteins (MAPs). Their functions are in a stabilising or regulatory role of the microtubule structure (Bray, 1992).

### **Assembly of Microtubules**

Microtubules are very dynamic structures constantly growing and depolymerising. Rapidly growing microtubules are more stable than slow growing ones and this is termed dynamic instability. Polarity is also found in the kinetic behaviour of microtubules; in most polymerising conditions, tubulin binds faster onto one end, termed the plus end than at the minus end (Bray, 1992). Tubulin dimers are added by the hydrolysis of the GTP molecule after the dimer has added to the microtubule. At rapidly growing ends, dimers attach faster than the GTP can be hydrolysed producing a GTP cap facilitating further attachment. A negative feedback mechanism at the level of protein synthesis in which free tubulin reduces the half-life of tubulin mRNA also controls microtubule levels within a cell (Bray, 1992).

### **Heterogeneity in Microtubules**

Variation in microtubule use suggests that microtubules may not all be identical. Most eukaryotes have small multigene families encoding both  $\alpha$  and  $\beta$  tubulin which can lead to different isoforms (Cleveland, 1987). Further heterogeneity can be introduced by various post-translational modifications of tubulin. These include addition of an acetyl group to, or the removal of a terminal tyrosine residue from  $\alpha$  tubulin (Bray, 1992).

## Microtubule Function

The function of microtubules within cells is varied. They can be found within three main types of structure; mitotic and meiotic spindles, cytoplasm, and ciliary axoneme of which their function during mitosis and meiosis is concentrated on in this study.

Mitosis like meiosis is divided into a number of stages according to the behaviour and position of the chromosomes. Whereas meiosis results in the reduction by half in the chromosome content and chromosome mixing in the daughter cells, mitosis is essentially replication; with daughter cells maintaining the chromosome number with no genetic changes. The function of microtubules will be discussed in terms of mitosis involving the stages prophase, metaphase, anaphase and telophase and these will be discussed in turn in relation to cytoskeletal mechanisms. Similar arrays of microtubules to those found in mitosis (discussed below) are used in meiosis to segregate chromosomes into germ-line cells and during fertilization to facilitate the fusion of male and female pronuclei (Bray, 1992).

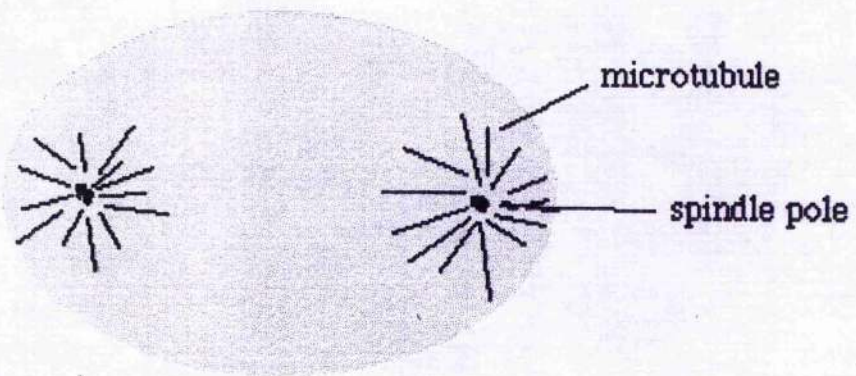
### *Prophase*

Just before the cell enters prophase, chromosomes condense and centrosomes outside the nuclear envelope duplicate producing two centres that move to opposite sides of the nucleus. These act as focal points for the microtubule array known as the mitotic spindle. The mitotic spindle forms by microtubule growth on the centrosomes. At the end of prophase the nuclear membrane breaks down and the microtubules enter the space previously occupied by the nucleus.

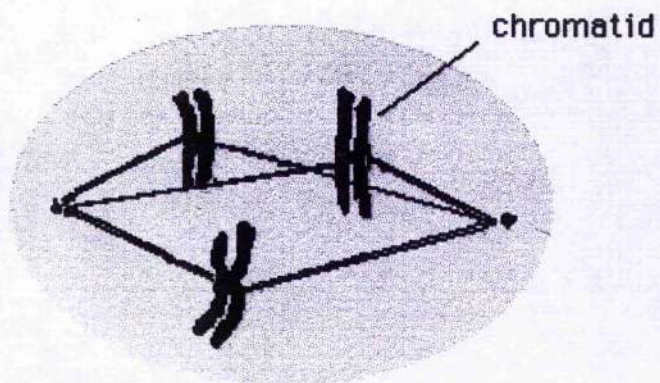
### *Metaphase*

Three types of microtubule structure (shown in Fig. 2.1) are produced as the cell enters the pro-metaphase stage: Polar microtubules grow together from opposite poles, astral microtubules have plus ends free in the cytoplasm and the kinetochore

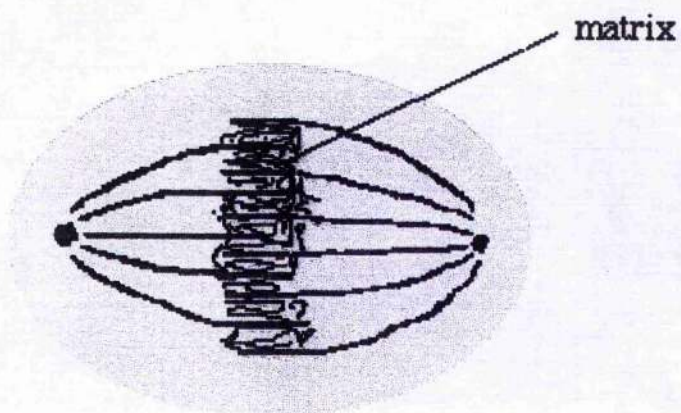




**Astral Microtubules**



**Kinetochore Microtubules**



**Polar Microtubules**

Fig. 2.1. Three categories of spindle microtubules at mitosis. The region of overlap of spindle microtubules may contain a matrix of microtubule-associated proteins (from Bray, 1992).

microtubules extend from the mitotic centres and attach to the kinetochore of each chromosome. Chromosomes are aligned on the metaphase plate by balancing forces of the kinetochore microtubules and polar microtubules. The metaphase stage can last 30 minutes.

### *Anaphase*

Chromosome centromeres split and separation of sister chromatids follows allowing the chromosomes to move apart. Movement is at about 1  $\mu\text{m}$  per minute toward the poles and is the result of two processes: the shortening of kinetochore microtubules by tubulin depolymerisation and elongation of the polar microtubules.

### *Telophase*

Chromosomes, drawn to separate poles, congregate into two new centres. Kinetochore chromosomes are no longer visible while polar microtubules are constricted by the contractile ring made up of actin and myosin. Nuclear membranes reform and the cells cleave and complete mitosis.

### **Labelling of Microtubules with Antibodies**

The labelling of microtubules with antibodies gives much more information than light microscopy and in oocytes with dense yolk it is the only way to visualise microtubule structures in whole mounts. The advantages over electron microscopy are that whole cell mounts can be made making the need for serial sections redundant.

Antibodies are host proteins produced in response to the presence of foreign molecules (antigens) in the body. It is the interaction of the antibody with an antigen that forms the basis of all immunochemical techniques. Antibodies bind specifically to the antigen at a specific site, termed the epitope. Antibodies, which are mainly  $\gamma$  globulin proteins, consist of a number of types of which the IgG class is the most common. Antibodies are raised by immunising host animals such as rabbits etc. with the antigen which may be a large protein, in which case it can be used alone. If it is a

small peptide, which may not be immunogenic, it would have to be coupled to a larger protein. The antibodies produced in the serum may not be specific to the required antigen and so the serum must be tested for specificity to the pure antigen. Within the serum, antibodies recognising the antigen may be produced, but from a number of different sources. All these antibodies have slightly differing affinities and specificities and are termed polyclonal antibodies. These have limited usage because of their heterogeneity.

The development of monoclonal antibodies allows the unlimited production of identical antibodies because they are synthesised by a population of identical cells. Each population is descended from a single hybridoma cell formed by fusing an antibody-producing cell with a tumour cell that has the capacity for unlimited proliferation. It is monoclonal antibodies that are of great value in immunocytochemistry and a specific antibody (DM 1A raised in mouse) is commercially available. This antibody labels general  $\alpha$  tubulin by binding to an epitope near the C terminus (Kreis and Vale, 1993).

In this study the indirect method of immunofluorescence was used to label the microtubules and was pioneered by Coons and Kaplan (1950). In this method the primary antibody, in this case DM 1A, binds to the specific epitope of fixed material. The addition of a second antibody raised to the  $\gamma$  globulin of the species which donated the first antibody binds to an epitope on the primary antibody. The secondary antibody is conjugated with a fluorescent label (FITC), allowing the structures to be visualised under the fluorescence microscope. In this example, sheep anti-mouse  $\gamma$  globulin binds to a specific epitope on the DM 1A. This method has advantages over the direct method as anti IgG sera have very high avidity and two labelled anti-immunoglobulin molecules can bind to each primary antibody increasing the sensitivity of the reaction.



## **Tubulin in the General Pattern of Development of Embryos**

In sea urchins, mature eggs have little or no tubulin synthesis but do contain a large store of unpolymerised tubulin of which 10% is incorporated into spindles and cilia, although the fate of the rest is unknown. At fertilization tubulin is polymerised from the pool of tubulin, but is also synthesised from maternal mRNA which is stored in the unfertilized egg (Alexandraki and Rudeman, 1985).

## **Microtubule Dynamics During Oocyte Maturation in Other Species**

In comparison to the microtubule dynamics during fertilization and cleavage of oocytes (see chapter 8) there have been relatively few studies of microtubule changes during oocyte maturation. Of these studies, there have been two approaches to the examination and characterisation of microtubule structures in oocytes. The first is to look at isolated microtubule structures *in vitro*. The second is to study microtubule structures in intact cells.

Isolated microtubule structures have been used in the assessment of total levels of tubulin and ratios of polymeric tubulin to tubulin dimer during the maturation of *Xenopus laevis* oocytes (Jessus *et al.*, 1987). The authors found that, although the total levels of tubulin remained unchanged, the level of polymeric tubulin decreased during the maturation process. Microtubules are also present in the prophase oocyte, although they are more stable than microtubules present in the metaphase oocyte. Two high molecular weight proteins were also found to be associated mainly with prophase microtubules.

Indirect immunofluorescence has been used to follow microtubule changes in a number of ascidian species (*Molgula occidentalis*, *Ciona savignyi* and *Halocynthia roretzi*). Fixation of whole oocytes was in cold methanol after extraction for 1-2 hours (Sawada and Schatten, 1988). Microtubule changes were followed through meiosis, fertilization and mitosis.

Although there is a loss of resolution because of the cell thickness in using whole mounts, the use of the confocal microscope in conjunction with normal fluorescence in this study has overcome this problem by enabling only in focus light to be detected from images of up to 200  $\mu\text{m}$  thick. This system can provide optical serial sections through an oocyte and these can then be reconstructed to provide three-dimensional images of an oocyte consolidating the results obtained with conventional fluorescence microscopy.

### **2.1.3 Changes in Microtubule Structures Associated with Different Maturation Methods**

Oocyte maturation in *Arenicola marina* results in the oocytes being held at the metaphase stage of the first meiotic division (Howie, 1961b). The mature oocytes are characterised by a metaphase spindle and corresponding chromosome formation, both of which can be labelled consistently as described in section 2.2. These two characteristics, thus enable them to be used as markers to see if different methods of maturation affect these structures. In this experiment oocytes of *A. marina* were matured in three ways: a) the *in vitro* incubation of oocytes in coelomic fluid with maturation inducing (CMF) activity to induce maturation; b) the injection of homogenised prostomia into females to induce the spawning process and coincident maturation of the oocytes and c) the natural spawning of females to obtain mature oocytes. The labelling of the meiotic spindle and chromosomes in mature oocytes can be used to observe any differences in the development of the meiotic metaphase spindle due to the method of maturing the oocytes. The possible differences in development due to different maturation methods may also manifest themselves at the post-fertilization stage which is covered in chapter 8.

## 2.2 Materials and Methods

### 2.2.1 Collection and Maintenance of Specimens

Mature *Arenicola marina* were collected by digging in sandy beaches at low water of spring tides. Collections were made from a number of field sites around the coast of the British Isles enabling gravid individuals to be collected during several months of the year (Sept.- Jan.). Collections were made from: Red Wharf Bay, Anglesey, North Wales, (Grid reference OS-114 545805), (September and October); West Sands, St Andrews, Fife, (Grid reference OS-59 503185) and Kingsbarns, Fife, (Grid reference OS-59 604124) (October and November). Collections during November and December were from the Eden Estuary, St Andrews, Fife (Grid reference OS-59 485185), and John Muir Country Park, Dunbar, Lothian, (Grid reference OS-67 643805 and 657788 respectively). The differences in spawning times of different populations permitted experiments to be carried out over an extended period. In all experiments the population with the most mature individuals was used.

Sampling methods varied according to the population density of individuals estimated from visible casts on the surface of the sand. At West Sands, St Andrews and Kingsbarns (sites with relatively low population densities) animals were collected by digging between the head hole and cast with a garden fork. The worms at these sites were found at a depth of approximately 20-30 cm. *Arenicola marina* from Dunbar, Bangor and the Eden estuary sites had much higher population densities. They were collected, again by digging with a garden fork, but by digging trenches and removing all worms found, usually within the top 10-20 cm.

All animals collected were placed in buckets with a small amount of sea water except for those collected from the Bangor site which were kept in individual plastic containers with a small amount of sediment. They were then transported back to the laboratory in an insulated cool box. Upon return to the laboratory, animals were kept

individually, either in polyethylene or polystyrene containers. This was to prevent any pheromonal or other interactions occurring between individuals which might trigger spawning. Each container contained 150 ml of filtered sea water and was changed every 2-3 days. All animals were maintained at a constant 10°C with ambient illumination. All animals were kept for a minimum of 24 hours before use in experiments, to allow the gut contents to be voided.

### 2.2.2 Fluorescence Labelling of Chromosomes

The fluorescence labelling of the chromosomes using Hoechst dyes was designed to permit visualisation of the maturational stage of oocytes to be assessed using *in vitro* fluorescence staining of the chromosomal material. Using these dyes to label mature and immature oocytes could also be used to assess the presence of maturation inducing factors.

Stock solutions of the Hoechst dyes 33342 and 33258 (1 mg.ml<sup>-1</sup>) (Sigma Co.) were prepared in distilled water and frozen at -20°C until required for use. From these, stock solutions of 10 µg.ml<sup>-1</sup> were prepared in twice filtered (0.2 µm) sea water (TFSW). Hoechst 33342 solution was used for incubations on living cells, and Hoechst 33258 was used to stain fixed material (see below). Hoechst 33342 (10 µg.ml<sup>-1</sup>) solution was added to oocytes in 96 well plates or Eppendorf tubes, depending on the experiment, to give a final stain concentration of 1 µg.ml<sup>-1</sup>. These were then incubated for 20 min at 20°C and the number of prophase oocytes versus metaphase oocytes was determined using a fluorescence microscope (see below).

#### Fixation of Oocytes for Hoechst 33258 Staining

Oocytes were fixed in 2% formaldehyde in 20 mM PIPES at pH 6.8 with 5 mM EGTA, 0.5 mM MgSO<sub>4</sub> and 0.1% Triton X-100 (see appendix) for 6 min at room temperature followed by 24 min at 0°C. Oocytes were then washed in TFSW, and 10 µl of Hoechst 33258 (10 µg.ml<sup>-1</sup>) was subsequently added to 90 µl oocyte suspension in TFSW to give a final concentration of Hoechst 33258 of 1 µg.ml<sup>-1</sup>.

## Fluorescence Microscopy

Oocytes from each experiment to be assessed for maturation were mounted on glass microscope slides with coverslips. They were viewed using a Leitz Dialux 20 microscope with a HBO 50 W/AC mercury short arc lamp with filter block A, exciting at 340-380 nm and suppressing at 430 nm and NPL FLUOTAR objectives of magnification of x25/0.55 and x40/0.7. Three sub samples were removed and 50 oocytes were counted from each sample. Oocytes that had been damaged either before or during processing were excluded from the analysis. Photographs were taken using 1600 ASA colour film (Kodak EPH P1600 X).

### 2.2.3 Sequential Observations of Oocyte Maturation using Microtubule and Chromosome Labelling

To examine changes in the distribution of microtubules during maturation, oocytes had to be fixed at specific times after the maturation process had been initiated. The number of time points required to obtain a complete investigation of the processes of maturation made the use of *in vivo* matured oocytes impractical. Consequently, oocytes were matured *in vitro* by incubation in coelomic fluid with maturation inducing activity (i.e. coelomic fluid with Coelomic Maturation Factor [CMF] activity) as described in section 3.2.

#### Experimental Design

Pooled oocytes from oocyte donors were incubated in coelomic fluid with maturation inducing activity at 10°C under natural illumination. Five microlitres of oocytes were removed at the following time intervals after incubation had begun; 0, 10, 20, 40, 60, 80, 100, 120, 140, 160 and 180 minutes. Once removed, oocytes were fixed immediately, processed for microtubule and chromosome labelling and examined as described below. Fifty oocytes were also assessed for their maturational state at each time interval.

## **Immunofluorescence Labelling**

### *Fixation of Oocytes and Embryos*

Oocytes and embryos were fixed at the required developmental stage or time in formaldehyde fix (see appendix) for 30 minutes at 4°C. To prevent the formaldehyde in the fixation solution interfering with the antibody labelling and to facilitate storage of the embryos at 4°C, without deterioration of the microtubule structures, 95% of the fixative was removed and replaced with non-fixing PIPES buffer (see appendix).

### *Preparation of Coverslips for use in Labelling Procedures*

Round 13 mm diameter coverslips, thickness No. 1 (Chance Ltd.) were used to mount the oocytes. Circular coverslips reduced the loss of embryos during the labelling process, were more easily rinsed and easier to handle than square coverslips. The first step in the procedure was to reduce the negative charge of the glass to facilitate adherence of the oocytes and embryos to the coverslips. To do this it was necessary to add 75  $\mu$ l of poly-L-lysine solution (Sigma Co.) to the coverslip. The poly-L-lysine solution was then allowed to evaporate by placing the coverslips under a normal 60 W light for approximately 30 minutes. This leaves a layer of poly-L-lysine solution with a slight positive charge to the coverslip and allowed the oocytes and embryos to adhere to the coverslip even during washing.

### *Addition of Specimens*

Coverslips were placed on parafilm squares (2 cm) in plastic petri dishes (50 mm diameter x 13 mm depth). The specimen suspension (250  $\mu$ l) was pipetted on to the coverslip. In some cases, where the specimens had been stored for a number of weeks, gentle mixing before applying the specimens broke up any aggregates that had formed during storage. The specimen suspension was left for 10 minutes to allow the embryos to adhere, 2 ml of PBS buffer (see appendix) was then pipetted around the edges of the parafilm. This gently immersed the specimens and rinsed off any traces



of formaldehyde. The rinse was left on for 15 minutes and then aspirated off. Coverslips were further dried with blotting paper, although they were never allowed to dry out completely.

#### *Labelling with the Primary Antibody*

The primary antibody used was monoclonal antibody (clone no. DM 1A) raised in mouse (Sigma Co.). Aliquots of 2  $\mu$ l were frozen at -20°C and defrosted as required. The antibody was diluted to the required concentration in 1% sheep serum (Scottish Antibody Production Unit) in PBS buffer. The sheep serum was frozen in 10  $\mu$ l aliquots and defrosted immediately before being diluted with the PBS buffer. The final primary antibody concentration used was 1/1000 as this concentration was found to give the optimal labelling of microtubule structures.

The primary antibody 1/1000 dilution (30  $\mu$ l) was pipetted on to the coverslip and left for 4 hours. This amount of primary antibody prevented the specimens from drying out over the 4 hour period and made sure that the meniscus covered all the embryos' surface. Lids of the petri dishes were replaced to maintain a humid atmosphere and the dishes were then covered to prevent direct sunlight heating or drying out the specimens. Although primary antibody attachment and detachment reaches equilibrium, 4 hours was found to be the optimum time for incubation, any longer increased evaporation and shorter times increased the incidence of poor labelling of specimens on a coverslip. After 4 hours the rinsing procedure described above was repeated.

#### *Labelling with the Secondary Antibody and Hoechst 33258*

After immersion in the rinse for 15 minutes the PBS buffer was aspirated off and 30  $\mu$ l of the secondary antibody was added as described for the primary antibody and then left for 2 hours before rinsing. The secondary antibody used was anti-mouse and raised in sheep with the fluorescent label (FITC) conjugated to it (Scottish Antibody Production Unit). The working dilution for the secondary antibody was

1/100 and it was diluted with 1% sheep serum in PBS buffer. After the rinse had been aspirated off, 20  $\mu$ l of Hoechst 33258 (1  $\mu$ g.ml<sup>-1</sup> in distilled water) was added for 2 minutes then aspirated off.

### *Mounting the Coverslips*

During the 2 minute incubation of the specimens with the Hoechst 33258, 6  $\mu$ l of mounting fluid (see appendix) was placed on a clean microscope slide. After the Hoechst stain was aspirated off, the coverslips were dried with blotting paper and then placed, specimen side down, on to the mounting fluid. To prevent drying, each coverslip was then sealed by careful painting of nail varnish to overlap the edge of the coverslip by approximately 1 mm. Once the nail varnish was dry, slides could be stored for a number of weeks in the dark at 4°C until ready for viewing.

### *Controls*

For all immunolabelling series a control was included. This involved the replacement of the primary antibody with 1% sheep serum in PBS buffer. Negative staining when viewing the specimens confirmed that the labelling observed in the series was as a result of the recognition of the  $\alpha$  tubulin by the primary antibody.

### *Visualisation of the Microtubule Structures*

The following 3 methods were utilised:

1. A Leitz Dialux 20 microscope as described in Section 2.2.2
2. Nikon Diaphot 2 inverted microscope with a HBO 50 W/AC mercury short arc lamp with filter block A, exciting at 340-380 nm and suppressing at 430 nm and NPL FLUOTAR objectives of magnification of x20/0.55 and x60/0.7. Photographs were taken using monochrome 400 ASA TMY film.
3. Confocal images: Confocal Laser Scanning Microscope with krypton/argon lasers (Bio-rad Microscience Ltd; MRC-600, Nikon Diaphot 2 inverted microscope as



above). Data sets were stored on 5 1/4" optical disks (Phillips) and copies of data were downloaded using a colour video printer (Sony VP5000) onto colour printer paper.

## **2.2.4 The Effect of Maturation Method on Microtubule Structure and Chromosome Arrangement**

### **Experimental Design**

The maturation of oocytes of *Arenicola marina* was induced in three ways, Treatments 1, 2 and 3;

#### *In vitro Incubation in CMF (Treatment 1)*

Coelomic fluid with CMF activity (see Chapter 3), was obtained by the injection of 2 females with 1 equivalent prostomium each and removed 2 hours post injection as described in section 3.2.1 and stored on ice until required. Coelomic fluid containing oocytes (100  $\mu$ l), was removed from each of 6 oocyte donors (1-6). Oocytes were allowed to settle out and then washed 3 times in TFSW. These oocytes were incubated in 50  $\mu$ l of coelomic fluid with CMF activity for 3 hours at 10°C under ambient illumination.

#### *Induction of Spawning by Injection of Homogenised Prostomia (Treatment 2)*

Once oocytes to be incubated in coelomic fluid with CMF activity had been removed from females 1-6, each of these females was then injected with 1 equivalent homogenised prostomia as described 3.2.1. They were then maintained at 10°C under ambient illumination and allowed to spawn at which time the spawned oocytes were harvested and oocytes were classed as 1a-6a. All females spawned within 3 hours after injection.

*Obtaining Mature Oocytes by Natural Spawning (Treatment 3)*

As spawning in the field approached, worms kept in the laboratory often began to spawn spontaneously. In some cases spontaneous spawning could be induced by changing the water in the worm's container. This provided a convenient means of obtaining naturally spawned gametes. Five females were induced to spawn naturally by changing the water. Five females spawned within 2-3 hours, the other female (female 7) had spawned overnight and the oocytes were approximately 18 hours old.

Once mature oocytes were obtained from the three methods described above, all oocytes were washed in TFSW and each set divided into two. One half was fixed immediately, processed for microtubule and chromosome labelling as described above. The other half was fertilized, as described in section 4.2.3, with a mixture of naturally spawned sperm from 3 males, to give a final concentration of  $10^4$  sperm per ml. Oocytes were incubated for 5 hours at  $10^{\circ}\text{C}$  under natural illumination before fixing as described above. Oocyte sets 1-6 (removed from females 1-6 and matured *in vitro*), 1a, 4a, 5a and 6a (mature oocytes obtained by injection of prostomial homogenate into females 1, 4, 5, and 6 which then spawned) and 7, 10, 11, 12 (mature oocytes obtained from naturally spawning females) were 1 hour younger when fertilized than the other sets of oocytes.

**Examination of Metaphase Spindle characteristics**

The criteria to look at any changes in the maturation characteristics due to differences in the maturation process were changes in the maximum diameter of the metaphase spindle and the ring of chromosomes both from a polar view. 25 oocytes from each female from each type of maturation were viewed using a Leitz Dialux 20 microscope as described above.

## 2.3 Results

### 2.3.1 Hoechst Staining of Immature and Mature Oocytes

The fluorescent assay clearly shows the differences between immature oocytes held at the late prophase stage of meiosis I and mature oocytes (in metaphase). In both cases the dye has bound to the DNA and stains the chromosomal material brightly in comparison to the remainder of the cytoplasm. Figure 2.2a shows an unfixed oocyte, in prophase I stained with Hoechst dye 33342, while Figure 2.2b shows a fixed oocyte, in prophase I stained with Hoechst dye 33258.sed. The remainder of the nucleus remains unstained. The chromosomes of these oocytes are dispersed and are not fully condensed. The position, shape and form of the chromosomes within the prophase nucleus can be visualised. The oocytes are characterised by the presence of the germinal vesicle, and are arrested at diplotene of late prophase. Figure 2.3a shows an unfixed mature oocyte stained with Hoechst dye 33342, while Figure 2.3b shows a fixed mature oocyte stained with Hoechst dye 33258. Both oocytes have undergone maturation and have moved from prophase to the metaphase stage of the first meiotic division. The nuclear membranes have disappeared showing that germinal vesicle breakdown (GVBD) has taken place. The repositioning of the chromosomes at metaphase can be seen. The condensed chromosomes are at the centre of the oocytes and they are aligned in a typical metaphase plate arrangement. The metaphase plates are seen from a polar view, with the chromosomes appearing in a circular (ring) arrangement in both figures, although not as clearly defined in Figure 2.3a as 2.3b .

Figures 2.4 and 2.5 are light micrographs of a prophase and a metaphase oocyte respectively. These demonstrate, how in some cases with normal light microscopic techniques, differences between immature and mature oocytes are not always obvious. In comparison, the visual differences in the prophase (immature) oocytes (Fig. 2.2a, b) and the metaphase (mature) oocytes (Fig. 2.3a, b) are striking,

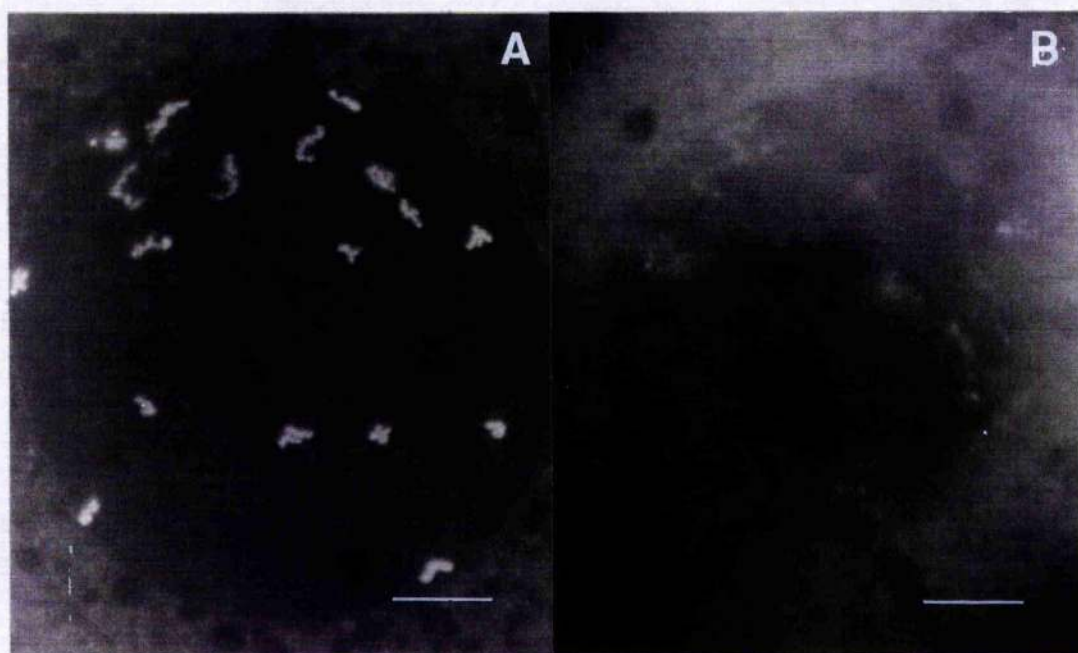


Fig. 2.2. Unfixed (Fig. 2.2a) and fixed (Fig. 2.2b) immature oocytes of *Arenicola marina* at prophase of first meiotic division, stained with Hoechst 33258 and 33342 respectively (final concentration  $1 \mu\text{g.ml}^{-1}$ ). Scale bar= $25 \mu\text{m}$ .

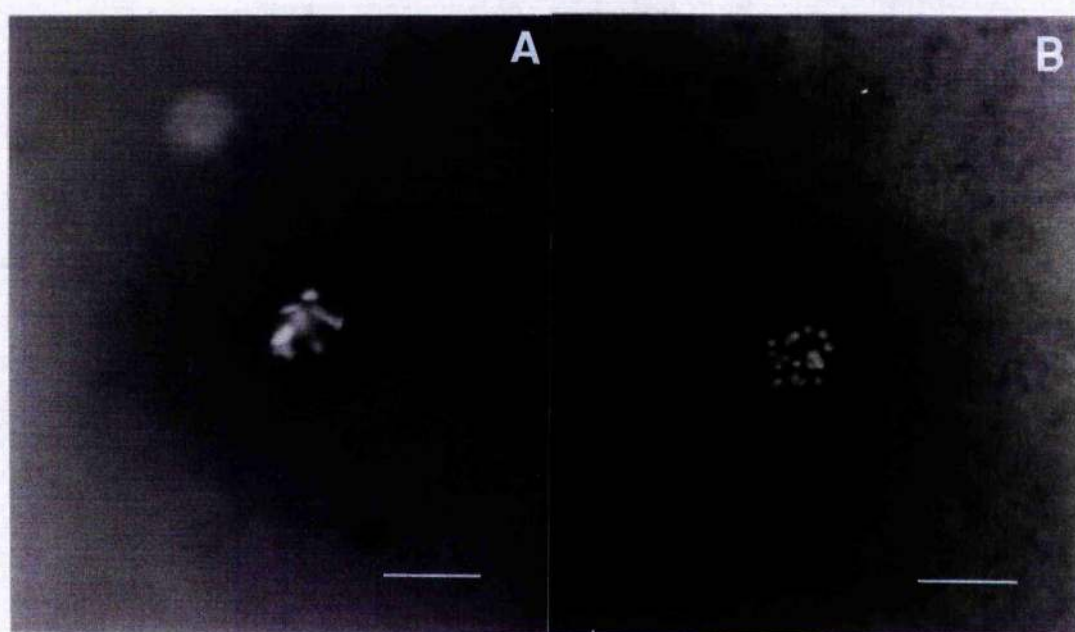


Fig. 2.3 Unfixed (Fig. 2.3a) and fixed (Fig.2.3b) mature oocytes of *Arenicola marina* at metaphase stage and stained with Hoechst 33258 and 33342 respectively (final concentration  $1 \mu\text{g.ml}^{-1}$ ). Scale bar=  $25 \mu\text{m}$



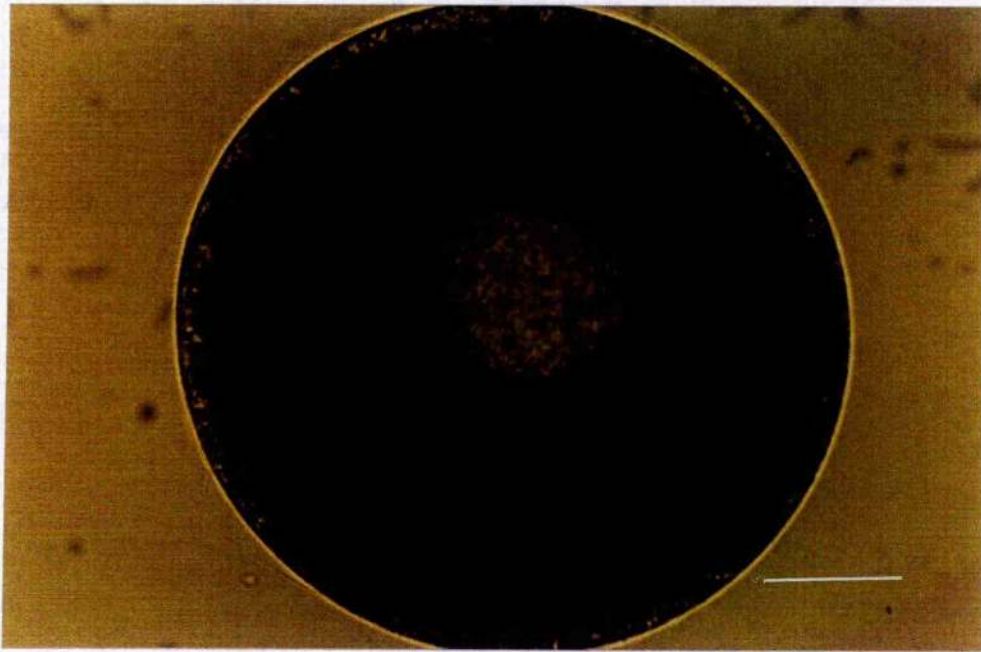


Fig. 2.4. Light micrograph of an immature *Arenicola marina* oocyte at prophase of the first meiotic division. Scale bar=50  $\mu\text{m}$ .



Fig. 2.5. Light micrograph of a mature *Arenicola marina* oocyte at metaphase of the first meiotic division. Scale bar=25  $\mu\text{m}$ .

demonstrating the ease with which maturational stages can be assessed. The use of fixed or unfixed material was dependent on the time constraints for each experiment only. This forms the basis for the *in vitro* assay for the coelomic maturation factor (CMF) described in section 3.2.

### **2.3.2 Sequential Observations of Oocyte Maturation using Microtubule and Chromosome Labelling: a Time Series**

Figures 2.6 to 2.12 show oocyte maturation in relation to the development of the meiotic spindle and corresponding chromosomal movements in *Arenicola marina*. In Figures 2.6-2.12 labelling of the microtubules is with the monoclonal antibody, DM 1A, as the primary antibody and the secondary antibody used was anti-mouse and raised in sheep with FITC conjugated to it. Corresponding staining of the chromosomes was with  $1 \mu\text{g.ml}^{-1}$  of Hoechst 33258. The microtubule labelling in the immature, prophase of meiosis I arrested oocyte (prior to incubation in CMF), is concentrated around the edge of the germinal vesicle in a tight band of  $10 \mu\text{m}$  width (Fig. 2.6a). It is likely that the microtubules cover the whole of the surface of the germinal vesicle but due to the focal plane limitations of the microscope can only be seen around the edges. The corresponding figure of the distribution of chromosomes (Fig. 2.6b) shows that at this stage individual chromosomes are very difficult to distinguish, but are non-condensed, and dispersed throughout the germinal vesicle.

As maturation proceeds there is little noticeable change in the distribution of microtubules or chromosomes until 40 minutes after the start of CMF incubation. At this time the distinct line of microtubules surrounding the germinal vesicle has begun to break up, although the germinal vesicle is still intact and visible (Fig. 2.7a). Microtubules are now spread across the centre of the germinal vesicle in no distinguishable pattern. The chromosomes at this stage have begun to condense and are now easier to visualise (Fig. 2.7b).



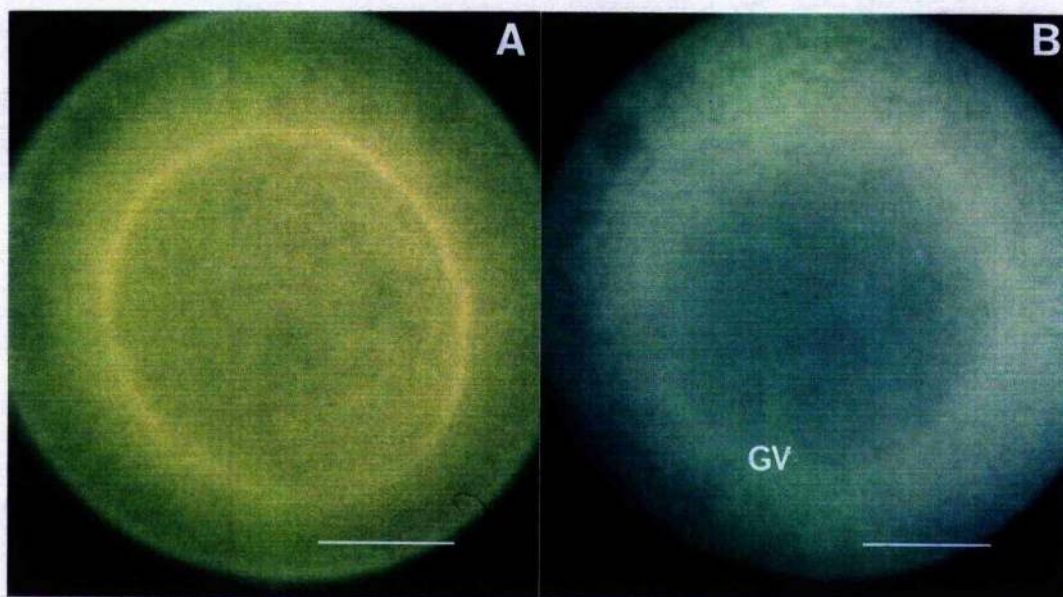


Fig. 2.6. Oocyte of *Arenicola marina* after 0 mins of CMF incubation. The microtubules are only visible in a tight (10  $\mu\text{m}$  diameter) band around the edge of the germinal vesicle (Fig. 2.6a). The chromosomes are very difficult to visualise as they are decondensed and spread throughout the germinal vesicle (GV) (Fig. 2.6b). Scale bar=50  $\mu\text{m}$ .

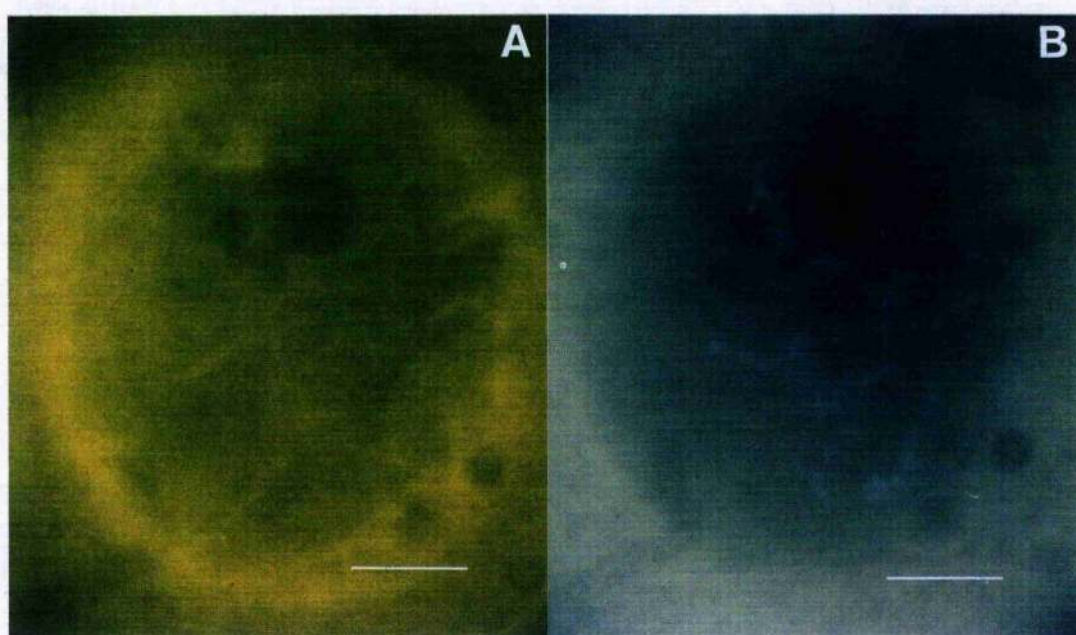


Fig. 2.7. Oocyte of *Arenicola marina* after 40 mins of CMF incubation. The distinct line of microtubules has broken down and microtubules have spread across the centre of the GV (Fig. 2.7a). Chromosomes have begun to condense (Fig. 2.7b). Scale bar=25  $\mu\text{m}$ .

After 60 minutes of incubation a distinct change can be seen in the arrangement of microtubules. The germinal vesicle has broken down and a mass of microtubule labelling in the centre of the oocyte is visible (Fig. 2.8a). The microtubules cover the area where the germinal vesicle was present, but do not seem to be in any regular pattern. The chromosomes at this stage have started to move towards the centre of the oocyte and are condensing further as they are brighter and easier to visualise (Fig. 2.8b).

In the majority of oocytes the meiotic metaphase spindle has formed after 80 minutes of incubation. The spindle consists of microtubules radiating out from a central point that corresponds to a polar view of the spindle (Fig. 2.9a). The polar view made it difficult to distinguish the three specific microtubule types; astral, kinetochore and polar, as described above. Figure 2.9b shows at this stage, chromosomes are continuing to condense, move towards and align themselves on the spindle.

From 100 minutes through to 180 minutes of incubation there were few obvious changes to the form of the spindle although there was a reduction in size (specifically diameter) in some cases. The changes that take place through this time period are the continued condensation and alignment of the chromosomes on the metaphase spindle (Fig. 2.10a,b). This figure is representative of a continual movement of the chromosome aligning themselves on the metaphase spindle. In some cases during preparation oocytes were squashed so that the metaphase spindle was seen from a lateral view (Figs 2.11a,b). In this oocyte the metaphase spindle can be seen to be made up of astral microtubules and the midzone consisting of polar and kinetochore microtubules. However, the levels of resolution make it impossible to distinguish whether microtubules were kinetochore (connected to chromosomes) or spindle microtubules.

After 180 minutes of incubation all oocytes observed from a polar view had reached the "ring stage". The meiotic spindle at this stage consists of a central,



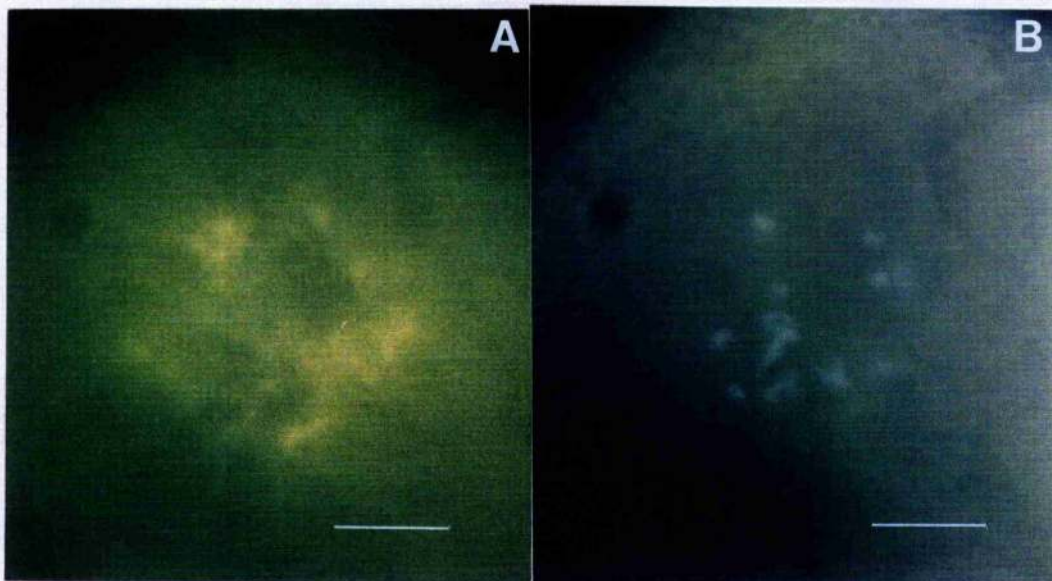


Fig. 2.8. Oocyte of *Arenicola marina* after 60 mins of CMF incubation. The germinal vesicle has broken down and microtubules are aggregated in the centre of the oocyte although in no visible pattern (Fig. 2.8a). The chromosomes have condensed further and are positioned in the centre of the aggregation of microtubules (Fig.2.8b). Scale bar=25  $\mu$ m.

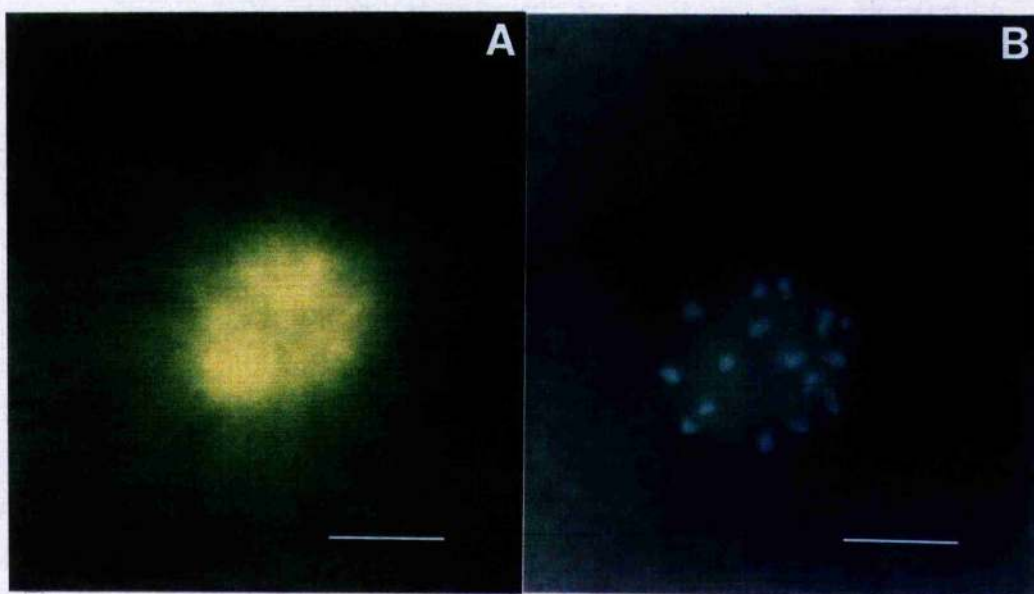


Fig. 2.9. Oocyte of *Arenicola marina* after 80 mins of CMF incubation. The meiotic metaphase spindle has formed. It consists of microtubules radiating out from a central point corresponding to a polar view of the spindle (Fig. 2.9a). The chromosomes continue to condense and begin to align themselves on the meiotic spindle (Fig. 2.9b). Scale bar=25  $\mu$ m.

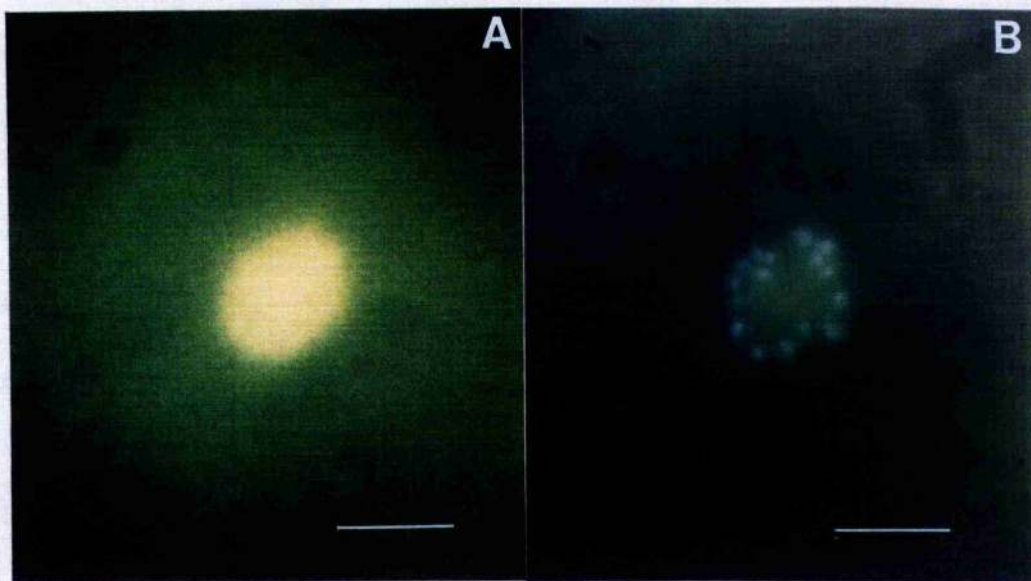


Fig. 2.10. Oocyte of *Arenicola marina* after 100-180 mins of CMF incubation. The meiotic metaphase spindle (Fig. 2.10a) with continued alignment of chromosomes on the spindle (Fig. 2.10b). Scale bar=25  $\mu$ m.

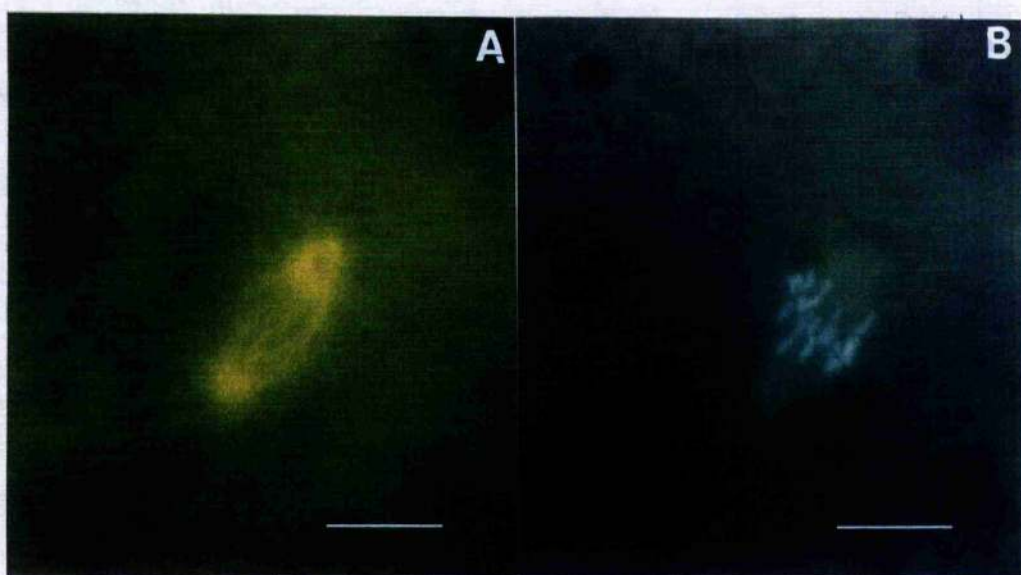


Fig. 2.11. Oocyte of *Arenicola marina* after 100-180 mins of CMF incubation. A lateral view of the meiotic metaphase spindle showing astral microtubules. The kinetochore and spindle microtubules are also visible but cannot be distinguished (Fig. 2.11a). The corresponding chromosomes can be seen aligning themselves in the centre of the metaphase plate (Fig. 2.11b). Scale bar=25  $\mu$ m.



circular area of microtubules radiating out towards the periphery (Fig. 2.12a). Chromosomes are arranged in a typical metaphase spindle arrangement; aligned in such a way as to form a ring of chromosomes (Fig. 2.12b).

From the fifty oocytes counted at each fixation time interval, it is possible to examine the kinetics of oocyte maturation and the development of the specific stages highlighted above. Figure 2.13 shows the general sequence of changes occurring during maturation in *Arenicola marina* oocytes both in terms of chromosomal and microtubule changes.

### **2.3.3 The Effect of Maturation Method on Microtubule Structure and Chromosome Arrangement**

The results of the measurements of maximum spindle and chromosome ring diameter in a polar view are shown in Figure 2.14. Statistical analysis using nested one way ANOVA was performed on the statistical package; Minitab ver. 8.2. With a significance level set at  $p$  is less than or equal to 0.05, analysis has shown that there were no significant differences between mean maximum diameters of metaphase spindles ( $F=1.57$ ,  $p=0.210$ ) or chromosome ring diameter ( $F=1.09$ ,  $p=0.336$ ) between the three treatments: *In vitro* incubation in CMF (Treatment 1); obtaining mature oocytes by injection of prostomial homogenate into females which then spawn (Treatment 2); and obtaining mature oocytes from naturally spawning females (Treatment 3). The method of maturation therefore has no effect on the diameter of the metaphase spindle and the diameter of the corresponding chromosome ring. A paired sample  $t$ -test confirmed that maximum chromosome ring diameter is significantly smaller than the maximum spindle diameter for all measurements ( $t=17.455$ ,  $p<0.001$ ).

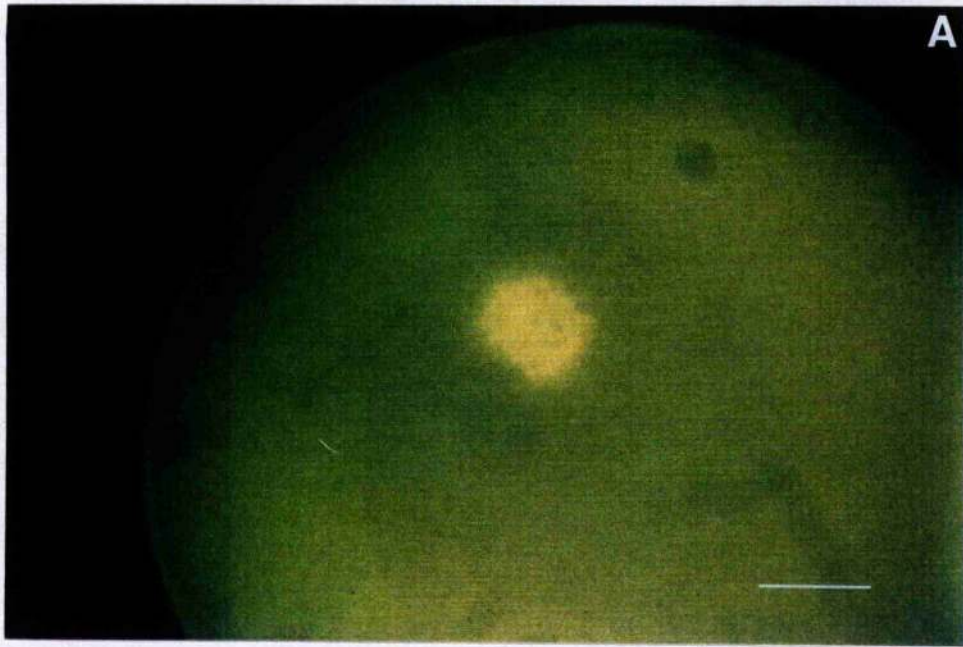


Fig. 2.12. Oocyte of *Arenicola marina* after 180 mins of CMF incubation. The meiotic metaphase plate is reduced in size (Fig. 2.12a) and chromosomes have completed movements and condensation into a typical "ring" alignment (Fig. 2.12b). Maturation is considered complete when oocytes have reached this stage. Scale bar=25  $\mu$ m.

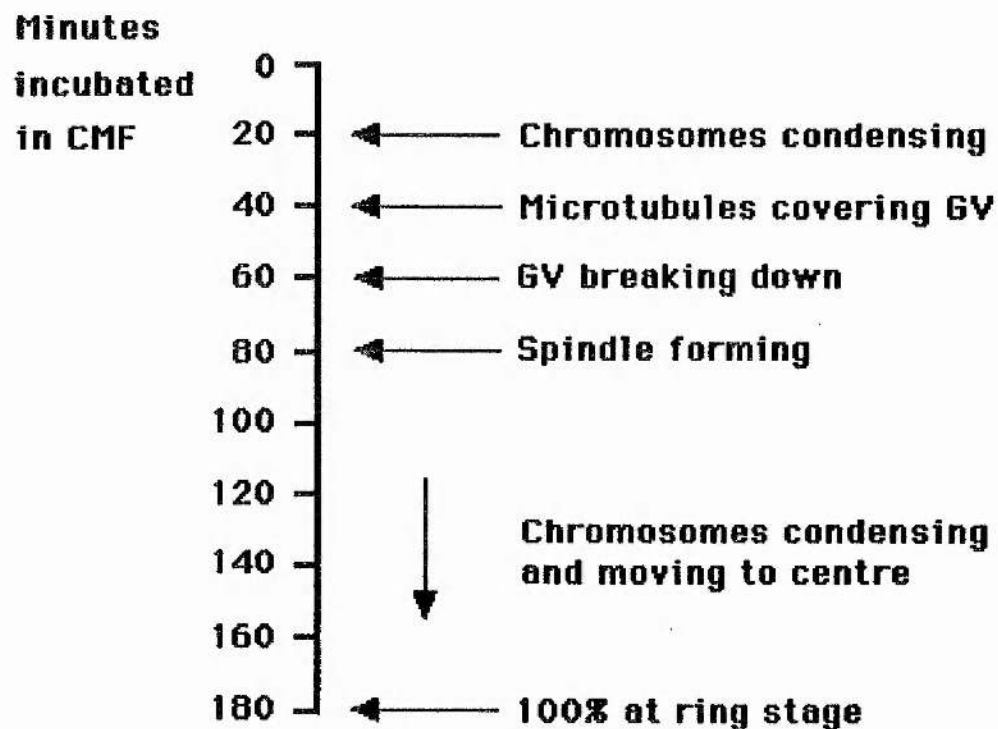
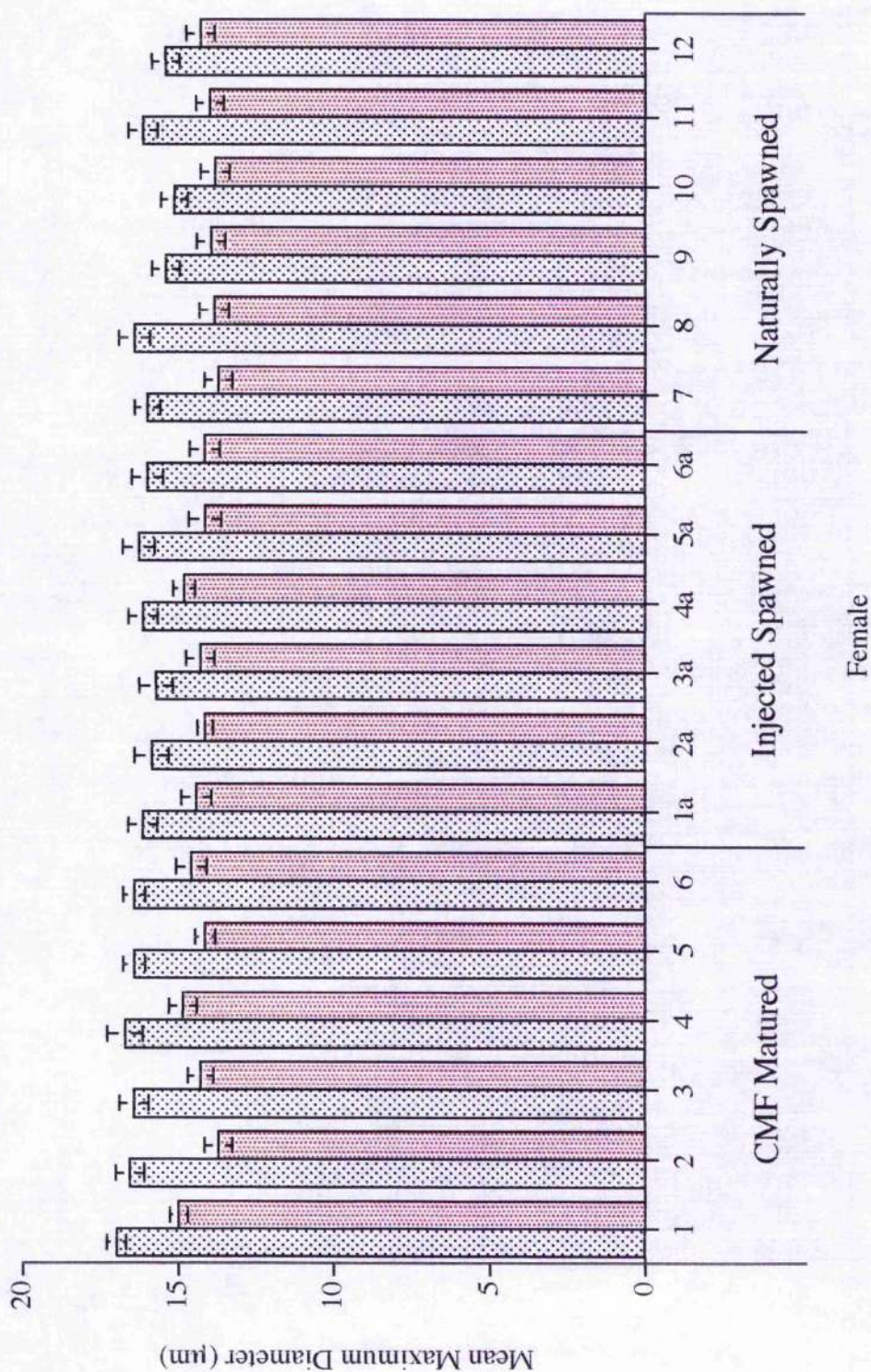


Fig. 2.13. Kinetics of oocyte maturation (GVBD) in relation to microtubule and chromosomal changes in oocytes of *Arenicola marina* after 3 hours of CMF incubation at 10°C. Times of specific morphological changes are averaged from 50 oocytes and are not exact. GV = Germinal Vesicle.

Fig. 2.14. Mean maximum diameters ( $\mu\text{m} \pm \text{S.E.M.}$ ) of polar view of metaphase spindle (grey stippled bars) and chromosome ring (red stippled bars) of first meiotic division of *Arenicola marina* oocytes after being matured with different treatments: CMF Matured, oocytes matured after incubation for 3 hours in coelomic fluid with CMF activity (Treatment 1); Injected Spawned, oocytes matured after being spawned by females injected with homogenised prostomia solution (Treatment 2); Naturally Spawned, oocytes matured after being spawned from naturally spawning females (Treatment 3). 25 oocytes counted per female. Oocytes from females 1-6 were removed for incubation in CMF prior to the injection of homogenised prostomia into those females and subsequent spawning to produce oocytes 1a-6a.







## 2.4 Discussion

The use of Hoechst dyes has facilitated the development of an *in vitro* fluorescence assay that has provided a quick and accurate means of assessing the maturation of coelomic oocytes of *Arenicola marina*. Conventional bright field microscopy does not allow reliable and rapid scoring of oocyte maturation (GVBD). An assay for maturation could be based on the ability of the oocytes to undergo fertilization as oocytes that have matured can be fertilized. However, this is not as rapid and also makes the assumption that oocytes that have undergone maturation will always fertilize (see section 3.4.1 for further discussion).

Although this is the first time that Hoechst dyes have been used to look at the control of oocyte maturation in *Arenicola marina*, they have been widely used in other invertebrate groups for a number of years. Neant *et al.* (1994), for example, used Hoechst 33242 to label chromosomal material in polar bodies of oocytes of the blue mussel (*Mytilus edulis*). This technique was used to look at the control of release from metaphase I by following the chromosomal material with the dye during the addition of protein synthesis inhibitors. Examination of the control of metaphase arrest using Hoechst dyes has also been carried out on *Patella vulgata* oocytes (Colas *et al.*, 1993). The properties of these dyes have given them a wide range of uses, and they are likely to be of continued value in the investigation of oocyte cytology, especially when maturational stages are difficult to visualise with normal microscopic techniques.

Immunofluorescent labelling has enabled oocyte maturation to be characterised in reference to the development of the meiotic spindle and concomitant movement of chromosomes during maturation. Previous work by Meijer (1979a) examined the kinetics of GVBD in *Arenicola defodiens* by incubating oocytes directly in prostomial homogenate, using transmission and scanning electron microscopy (T.E.M. and S.E.M. respectively) and light microscopic techniques to examine morphological

changes. Meijer (1979a) made a detailed study through maturation and did observe microfilaments and the formation of the meiotic spindle. Nevertheless, sectioning of material meant that a whole picture could not be observed and whole spindles and their development could not be followed. The technique of immunofluorescence utilised here provides not only a holistic picture, but also focuses specifically on the microtubules and chromosomes.

Meijer (1979a) first visualised the presence of microfilaments in *Arenicola defodiens* around the edge of the germinal vesicle after 25 minutes, and by 30 minutes tubules had formed with GVBD beginning within 30 to 35 minutes. At 40 minutes the mixing of cytoplasm and nucleoplasm signified that GVBD was in progress and at this time condensing chromosomes could also be visualised. The formation of the meiotic spindle by the 45th minute and the end of microvilli withdrawal signified the end of maturation at 60 minutes. In contrast to Meijer's work, condensing chromosomes were first observed 20 minutes into incubation in this study. Microtubules appeared at around 40 minutes, covering the GV. At 60 minutes GVBD was signified by the loss of the nuclear envelope, the spread of spindles across the GV and their arrangement into a spindle formation. The meiotic spindle had formed at around 80 minutes, although in some oocytes the chromosomes continued to condense and move until 180 minutes when they reached their final positions on the metaphase plate.

A comparison of the time course of meiosis between these two or other species is problematic because of the arbitrary nature of the stages imposed on a continuous process. The situation is further complicated by the different methods of observation between this study and Meijer's work. S.E.M. and T.E.M. recognise more detail, but do not focus specifically on the microtubules and chromosomes. However, there are specific events that are clearly observable and distinguishable enabling some comparisons to be formulated between the two studies. The formation of the meiotic spindle for example can be seen in both studies, and takes twice as long to form in

*Arenicola marina* than in *A. defodiens* (40 minutes and 80 minutes respectively). The breakdown of the germinal vesicle begins after 30 minutes in *A. defodiens* but again takes twice as long in *A. marina*. The time taken for oocyte maturation in *A. marina* may be up to double the time taken in *A. defodiens*. The total time required for completion of maturation is also difficult to compare between species as the exact end of both meioses cannot be compared. In this study maturation was assumed complete when chromosomes had finished condensing and reached their final destination on the metaphase plate, and in some cases this was only after 180 minutes incubation. Meijer's examination of maturation did not follow the continued condensation of chromosomes and movement to the metaphase plate and so direct comparisons cannot be made.

There may be a number of explanations for such an apparent difference in the speed of maturation between the two species, although comparisons should be interpreted with caution. It may be that the two different species do have different maturation times. In other phyla oocyte maturation does vary even between members of the same phylogenetic class. The relative times required for oocytes to reach different stages of maturation, however, are fairly constant among species so that times to reach metaphase I for example are fairly similar (Masui and Clarke, 1979). The differences observed here between *Arenicola defodiens* and *A. marina* may therefore be due to other factors. Firstly, the *in vitro* incubation of both sets of oocytes in maturation inducing substances (CMF for *A. marina* and prostomial homogenate for *A. defodiens*) may result in different maturation speeds. This is because the mechanisms of maturation, which are at this time unknown, may be completely different. Secondly, the temperature of incubation for this study was 10°C which may be different from an unknown temperature of incubation for Meijer's work. Finally, oocyte populations exhibit a lack of synchrony between different females, but also within females (pers. obser.) which may accentuate any small differences in speeds of maturation.

This study has shown for the first time in *Arenicola marina* oocytes the specific microtubule organisation and corresponding chromosomal movement during the development of the meiotic spindle. Observations on microtubules during maturation in other species, especially invertebrates, are limited.

In the ascidian, *Molgula occidentalis*, GVBD is induced by mechanical removal of the oocytes. GVBD begins within 1-5 minutes after removal, is completed within 15 minutes, and is then arrested at the first metaphase of meiosis until fertilization (Sawada and Schatten, 1988). Observations from this study show that changes in microtubule structure during oocyte maturation in *M. occidentalis* showed a similar pattern to that of *Arenicola marina*: In both species, the contour of the GV was clearly distinguishable by the band of labelling around the edge of the GV. However, the network of microtubules filling the GV in *M. occidentalis* was not observed in *A. marina*. Once GVBD begins, microtubules were present in the post-GV area in both species and the meiotic spindle forms and chromosomes condense in similar ways. In *M. occidentalis* the meiotic spindle then aligns itself parallel to the oocyte surface at the animal pole prior to fertilization. After fertilization or activation the spindle then rotates into a perpendicular position, the timing of this is thought to be during, or shortly after, the cortical contraction (Sawada and Schatten, 1988). This is in contrast to the results presented here which suggest that in *Arenicola marina* the spindle aligns itself perpendicular to the surface immediately. This is also the case in the polychaete *Chaetopterus* sp. where an asymmetric meiotic spindle orientates perpendicular to the cell cortex at the animal pole (Lutz *et al.*, 1988).

In *Xenopus laevis* the formation of the first (M1) and second (M2) meiotic spindles and associated chromosome movements have been examined in detail by a number of authors. Gard (1992) using indirect labelling of methanol fixed oocytes, and observing live oocytes injected with fluorescein conjugated tubulin, under the confocal microscope, examined both meiotic spindles. He found that M2's rate of spindle elongation was faster than M1. Both spindles also shared a number of

common stages including i) formation of a compact aggregate of microtubules and chromosomes; ii) reorganisation of this aggregate resulting in the formation of a short bipolar spindle; iii) rotation of the spindle in alignment with the oocyte axis. Coincident with GVBD, a transient microtubule array was assembled from a disk shaped microtubule organising centre (MTOC) formed near the basal region of the GV (Huchon *et al.*, 1981; Jessup *et al.*, 1986; Gard 1992). It then rapidly migrated from its original site of assembly toward the animal pole of maturing oocytes (Huchon *et al.*, 1981). In *Arenicola marina* the meiotic spindle also forms a compact aggregate of microtubules and chromosomes, although other changes associated with maturation were not measured in this study.

The mean maximum diameter of the spindle in *Arenicola marina* was found to be significantly larger than the mean maximum diameter of the chromosome ring. This is what would be expected because a measurement of the spindle in a polar view would measure the astral spindles which radiate out furthest from the centre. The chromosomes are connected to the kinetochore spindles that do not radiate from the poles as the astral spindles do.

In terms of structural (diameter) changes to the meiotic spindle and consequently the chromosomal positions, different methods of maturing the oocytes fails to elicit any significant change. This is, perhaps, not surprising because different methods of maturation are likely to be only differences in where the start for maturation is initiated. For example, *in vitro* maturation using Coelomic Maturation Factor (CMF) from other females (Treatment 1) bypasses the natural production of both CMF and prostomial maturational substance for that worm so inducing maturation from CMF onwards. Mature oocytes obtained by injection of prostomial homogenate into a worm which then spawns (Treatment 2) results in the bypass of the production of the worm's own prostomial maturational substance triggering instead the production of its CMF only. Oocytes from naturally spawned females (Treatment 3) are exposed to the worm's own whole system as it produces its own prostomial



maturation substance followed by the production of CMF. In each case it is the trigger upstream from cellular events within the oocyte that are altered so that the processes of maturation within an oocyte are likely to remain unaffected. This is because the outcome for all treatments is the same regardless of the source of CMF, that is CMF acts on the oocyte to start the maturation system within that oocyte.

Studies of the effects of maturation methods on the morphology of the meiotic spindle are scarce in other invertebrates. A study on three species of ascidians, however, showed that formation of the first meiotic spindle in *Molgula occidentalis* was different from the other two species; *Ciona savignyi* and *Halocynthia roretzi*. In *C. savignyi* and *H. roretzi* both had meiotic spindles with well focused poles. In *M. occidentalis* the meiotic spindle was a small spindle like structure (Sawada and Schatten, 1988). The authors suggest that the differences in meiotic spindles are due to the different methods of maturation: mechanical activation of GVBD in *M. occidentalis* compared to natural spawning in the other two species. However, no direct evidence for this supposition exists and in *M. occidentalis* the spindle reverted to the normal structure upon fertilization suggesting that this may be an artefact of the fixation protocols used.

The absence of observed differences in the structure of the meiotic spindle with different maturation methods in *Arenicola marina* is in accordance with studies in mammals. No significant differences were observed in the ultrastructure of mouse oocytes matured *in vitro* and *in vivo* (Calarco, 1972; Calarco *et al.*, 1972; Szollosi *et al.*, 1972).

## **Chapter 3**

### **The Presence of Maturation Inducing Activity (CMF) in Coelomic Fluid and the Relationship Between Oocyte Maturation and Spawning**



### 3.1 Introduction

The process of oocyte maturation in *Arenicola marina* has been discussed in detail in Chapter 1. Previous experiments have shown that spawning in female *A. marina* can be induced by a substance from the prostomium (Howie, 1963; 1966; Pacey and Bentley, 1992a). Spawning can be induced artificially by injecting homogenised prostomia from other mature females into gravid females (Howie, 1961b; Pacey and Bentley, 1992a). Initiation of the spawning process by the putative maturation hormone results in the maturation of the oocytes and their subsequent shedding from ciliated funnels of the nephromixia (Howie, 1961a,b).

Evidence to date suggests that *A. defodiens* has a single step maturation process involving a substance from the prostomium acting directly on the oocyte. In contrast, oocyte maturation in *A. marina* requires a second maturation inducer in the coelomic fluid (Auckland, 1993; Watson and Bentley, 1996).

Section 3.2 describes the use of an *in vitro* assay to assess the maturational state of oocytes incubated in coelomic fluid from females after injection with prostomial homogenate or TFSW. The injection of females with prostomial homogenate induces the production of maturation inducing activity within the coelomic fluid. This second factor has been named the Coelomic Maturation Factor (CMF). The data presented here show that production of CMF can be induced routinely and its presence or absence can be assessed by the maturation of oocytes from other females.

The link between the production of prostomial maturation substance, CMF, the maturation of oocytes, and spawning is not fully understood. Howie (1966) showed that the substance in the prostomium that induces spawning is produced only during the breeding season; as the spawning season approaches more females respond to injection by spawning. This spawning response to injection of homogenised

prostomia was shown to be all or nothing (Pacey and Bentley, 1992a). Howie (1961b) also showed that during the breeding season only mature oocytes are accepted by the nephromixia and spawned.

There are four known different components of the spawning system; the production of prostomial homogenate, the production of CMF, the maturation of the oocytes, and their acceptance by the nephromixia. Each may be activated individually but this may not always activate the following step. Prior to the breeding season and in some cases during the breeding season animals injected with homogenised prostomia fail to spawn (Howie, 1966; pers. obser.). Howie (1966) failed to show whether a failure to spawn was; a) the result of the whole system not being initiated or b) the spawning process being initiated but failing at one of the subsequent stages. The experiments performed in section 3.3 address this problem; that is, if injection of prostomial homogenate can initiate the system, is it possible for it not to proceed? The experiments examine whether the system can, once initiated, be arrested specifically at the final stage where oocytes have matured but cannot be accepted by the nephromixia. The oocytes would remain in the coelom because the worms' nephromixia system is not fully competent to accept oocytes.

## **3.2 Maturation Inducing Activity of Coelomic Fluid; the Presence of CMF**

### **3.2.1 Materials and Methods**

For all the following sections in this chapter, *Arenicola marina* were collected and maintained as described in section 2.2.

#### ***In vitro* Assay for Maturation Inducing (CMF) Activity**

The basis for all the experiments requiring or investigating maturation inducing (CMF) activity of coelomic fluid is that oocytes from gravid oocyte donors (in which

all oocytes are immature) can be used to determine the maturation inducing activity of coelomic fluid. To induce the production of CMF and incubate oocytes in this coelomic fluid so that they mature requires a number of steps:

### **Removal of Gametes**

All individuals collected were sexed by light microscopic examination of coelomic contents following removal of approximately 20  $\mu$ l of coelomic fluid. Samples of coelomic fluid were removed using a 1 ml disposable syringe fitted with a 25 g hypodermic needle. Penetration of the body wall was usually at the intersection of the trunk and tail. In some cases, worms could be sexed without the need for removal of coelomic fluid as eggs or the milky sperm suspension were visible through the body wall especially in the tail region.

### **Induction of Spawning**

To induce the production of CMF in females the spawning process must be initiated and this can be induced by the injection of homogenised prostomia from other females into the body cavity of the female (Howie, 1963; Pacey and Bentley, 1992a).

#### *Preparation of Prostomial Homogenate for Injection*

Prostomia were ablated from gravid specimens of *Arenicola marina* using fine forceps and bow iridectomy scissors. Animals for prostomium removal were kept in a small amount of sea water allowing the prostomia to be everted naturally and visualised before ablation. The fine forceps were then used to hold the base of the prostomium and this was then excised using the bow scissors. Surplus tissue around the prostomium was excised and removed prostomia were placed in twice filtered (0.2  $\mu$ m) sea water (TFSW). Prostomia to be used immediately were stored on ice and homogenised at 0°C by sonication in a MSE Soniprep 150 ultrasonic disintegrator. Prostomia could also be stored whole in TFSW at -20°C for future use.

The concentration of prostomial homogenate used for injection is expressed as the number of prostomia that would be present in a 1 ml volume. Therefore, 5 prostomia homogenised and made up in a final volume of 5 ml is equivalent to 1 prostomia per ml of homogenate (or 1 prostomia.ml<sup>-1</sup>).

#### *Injection of Prostomial Homogenate*

Prostomial homogenate was made up to a final volume of 200  $\mu$ l and injected into the body cavity of the worm, where the trunk meets the tail, to give a final dose of 1 prostomium equivalent per individual. For control injections 200  $\mu$ l of TFSW was injected into the worm. Once injected, the worms were returned to their containers and maintained at 10°C under ambient illumination.

#### **Removal of Coelomic Fluid**

Removal of coelomic fluid with maturation inducing activity was carried out after the spawning process had been initiated. Harvesting of the coelomic fluid was carried out c. 2 hours from initiation of spawning or, when microscopic examination of coelomic oocytes showed them to be >90% mature. Removal of coelomic fluid often resulted in the appearance of blood in the coelomic fluid. This did not appear to have any adverse effects on oocytes incubated in this coelomic fluid, although the effects of blood on maturation inducing activity of the coelomic fluid (especially enzymatic effects) are hitherto unknown. Large females (collected from West Sands, St Andrews and Kingsbarns) could yield up to 10 ml of coelomic fluid while equally mature females from Dunbar would sometimes give only 1 ml of coelomic fluid because of their small body size. For some experiments, this resulted in pooling of coelomic fluid samples from a number of females to provide sufficient active coelomic fluid for the experiment. As maturation inducing levels varied between females' coelomic fluid at a given time, it was possible that pooled coelomic fluid might vary in the degree of maturation inducing activity. This could lead in some cases to an overall reduction in maturation inducing levels in the pooled coelomic fluid.

Once removed, the coelomic fluid was centrifuged for 30 seconds at 6500 rpm to sediment any oocytes and cell debris present. The supernatant was then removed and stored on ice until required. All fresh coelomic fluid was used within 0.5 hours of removal from the worm because of the unstable nature of maturation inducing activity present.

### Collection and Processing of Oocytes

Females that had not been used for any prior experimentation were selected for use as oocyte donors. Oocytes were removed from the coelom using a 1 ml syringe with a 25 g hypodermic needle and checked for a homogeneous population of immature oocytes. Oocytes were sedimented in Eppendorf tubes and the coelomic fluid supernatant was removed. The oocytes were then resuspended and washed 3 times in TFSW and kept at 4°C before use in the *in vitro* assay. In cases where the chemical or substance to be tested for maturational activity was in short supply and individual differences between oocyte donors were not important, the oocytes from a number of females were pooled.

### Experimental Design

Two experiments were carried out; the second experiment in 1995 was a repeat of that carried out in 1994, except that a prostomial homogenate concentration of 1.0 prostomia.ml<sup>-1</sup> was assayed for maturational activity instead of 0.2 prostomia.ml<sup>-1</sup>. For each of the two experiments, six individuals (1-6 in 1994 and 1a-6a in 1995) were injected with prostomial homogenate to induce spawning while a further six per experiment (7-12 in 1994 and 7a-12a in 1995) were injected with TFSW as described above. Oocytes from six additional independent females (a to f in 1994 and g to l in 1995) were used to assess maturation inducing activity of each of the 12 females' coelomic fluids. Oocytes were also incubated in prostomial homogenate directly and TFSW as positive and negative controls respectively. Assessment of maturation inducing activity was carried out using the fluorescence assay as described in section

2.2.2. Fifty oocytes were assessed for maturation in 1994 (experiment 1) and three counts of 50 oocytes each were assessed for maturation in 1995 (experiment 2) and any following experiments. All oocytes were unfixed when assessed for maturation and were subsequently stained with Hoechst 33342.

Replicate samples (50  $\mu$ l) of each solution to be tested for maturation inducing activity were pipetted into individual wells of a 96 well plate. Oocytes (3-5  $\mu$ l) from each of the oocyte donors was added to each treatment and the plates (or Eppendorf tubes) were incubated for 3 hours at 10°C under ambient illumination except where stated. A flow diagram of the protocol is shown in Figure 3.1.

#### *Treatment 1*

Spawning was initiated in 6 females by injection of homogenate containing 1 prostomium equivalent per female as described in section 3.2.1. Coelomic fluid was removed after 1.5 hours to interrupt the spawning process as described above.

#### *Treatment 2*

The six females each injected with 200  $\mu$ l of TFSW, as a negative control, had their coelomic fluid removed and processed as for the coelomic fluid from females of Treatment 1.

#### *Treatment 3*

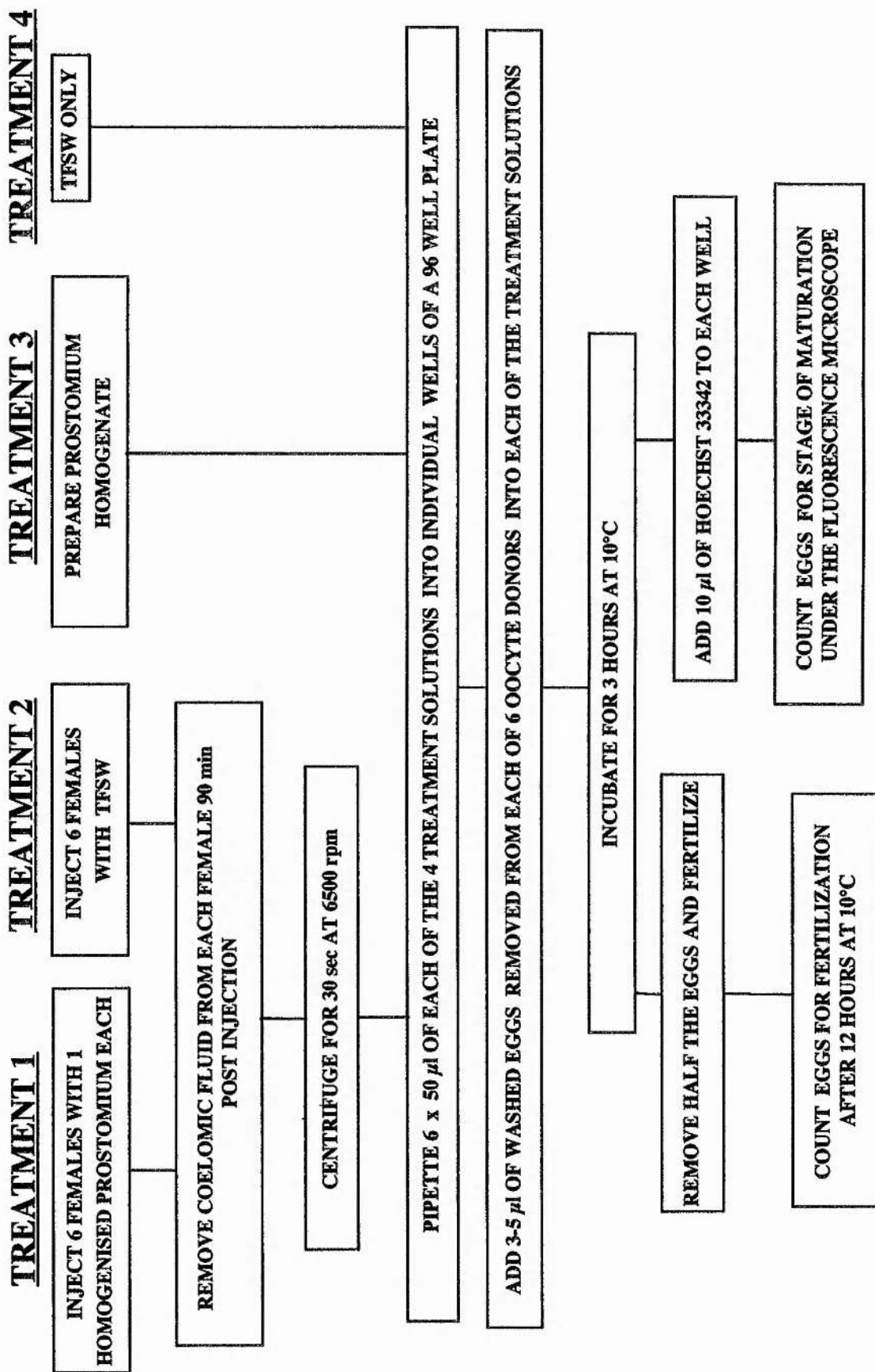
Prostomial homogenate was prepared as described above but at a concentration of 0.2 prostomia.ml<sup>-1</sup> (1994, experiment 1) and 1.0 prostomia.ml<sup>-1</sup> (1995, experiment 2) and assayed *in vitro* for the presence of maturational inducing activity as described above.

#### *Treatment 4*

TFSW was used as an *in vitro* negative control to test for spontaneous maturation of oocytes in both experiments.

Fig. 3.1. A flow chart showing the protocol to confirm the presence of CMF in coelomic fluid.





### *Fertilization*

After incubation, half of the eggs from each well were removed and placed in 1 ml of TFSW in an Eppendorf tube to which 10  $\mu$ l of active sperm was added. Fifty oocytes were assessed for fertilization in 1994 (experiment 1) and three counts of 50 oocytes each were assessed for fertilization in 1995 (experiment 2). The remaining oocytes were processed for percentage maturation using the fluorescence assay as described in section 2.2.2.

### **Statistical Analysis**

Statistical analysis was carried out using generalised linear interactive modelling (GLIM Version 3.77). This allows the distribution of non-normal data to be specified for analysis of variance. In 1994 (experiment 1) interaction effects could not be modelled adequately due to overparametisation of data. In 1995 (experiment 2) significant interaction effects were also present, but the data sets could support these extra parameters and so the models were applied with interaction included. To compare differences between the number of oocytes maturing and numbers that fertilized for each experiment, a Wilcoxon Signed Rank Test was performed on the pooled data.

### **3.2.2 Results**

The results of *in vitro* assays for CMF (maturation inducing) activity of coelomic fluid (obtained from individuals in which their endocrine status had been manipulated) and from control solutions are shown in Table 3.1. In both experiments (1994 and 1995) each of the 4 treatments was assayed for maturation using the *in vitro* fluorescence assay described above. Coelomic fluid samples 2-6 (1994) and 2a-6a (1995) have maturation inducing activity present and induced maturation of oocytes from all of the 6 females in both experiments. All other treatments from both experiments and coelomic fluid samples 1 and 1a have no maturation inducing activity present.

Treatment	Sample	Oocyte Samples					
<b>1994 (Experiment 1)</b>		<b>a</b>	<b>b</b>	<b>c</b>	<b>d</b>	<b>e</b>	<b>f</b>
Treatment 1	1	-	-	-	-	-	-
	2	+	+	+	+	+	+
	3	+	+	+	+	+	+
	4	+	+	+	+	+	+
	5	+	+	+	+	+	+
	6	+	+	+	+	+	+
Treatment 2	7-12	-	-	-	-	-	-
Treatment 3	PH	-	-	-	-	-	-
Treatment 4	TFSW	-	-	-	-	-	-
<b>1995 (Experiment 2)</b>		<b>g</b>	<b>h</b>	<b>i</b>	<b>j</b>	<b>k</b>	<b>l</b>
Treatment 1	1a	-	-	-	-	-	-
	2a	+	+	+	+	+	+
	3a	+	+	+	+	+	+
	4a	+	+	+	+	+	+
	5a	+	+	+	+	+	+
	6a	+	+	+	+	+	+
Treatment 2	7a-12a	-	-	-	-	-	-
Treatment 3	PHa	-	-	-	-	-	-
Treatment 4	TFSWa	-	-	-	-	-	-

Table 3.1. Maturation response of *Arenicola marina* oocytes (samples a to f, 1994; g to l, 1995) incubated in different treatments. Results are from two separate experiments using different material but the same protocol carried out in 1994 and 1995. Coelomic fluid samples from 6 females (1-6, 1994 and 1a-6a, 1995) removed 90 min after spawning had been initiated in each female by injection of 1 equivalent homogenised prostomia from other females (Treatment 1); coelomic fluid samples from 6 females (7-12, 1994 and 7a-12a, 1995) removed 90 min after injection of 200  $\mu$ l of TFSW (Treatment 2); PH, prostomium homogenate (0.2 prostomia.ml<sup>-1</sup>, 1994) and PHa, prostomium homogenate (1.0 prostomia.ml<sup>-1</sup>, 1995) (Treatment 3); TFSW, TFSW only, 1994 and TFSWa, TFSW only, 1995 (Treatment 4). All incubations were carried out at 10°C for 3 hours. + = mature oocytes present; - = no mature oocytes present.

In 1994 (experiment 1) there is no significant inter-individual variation in the maturational ability of oocytes from different oocyte donors ( $F=3.04$ ,  $p>0.05$ ). In 1995, however, there are significant differences in the ability of oocytes of the six oocyte donors to undergo maturation ( $F=57.04$ ,  $p<0.001$ ). Interaction between oocyte donors and coelomic fluid samples for the presence of maturation inducing activity (percentage number of oocytes maturing) in 1995 (experiment 2) is also highly significant ( $F=8.30$ ,  $p<0.001$ ).

Figure 3.2 shows the mean percentage number of mature oocytes (stippled bars) after incubation in coelomic fluid of females 1-6 (1994) and 1a-6a (1995). Only in coelomic fluid from females 2-6 and 2a-6a (Treatment 1) where the spawning process had been initiated is there significant maturation inducing activity. There are significant differences between the ability of coelomic fluid from females 2-6 and between coelomic fluid from females 2a-6a to induce maturation of oocytes, ( $F=14.63$ ,  $p<0.001$ ) and ( $F=22.37$ ,  $p<0.001$ ) respectively. Both females 1 and 1a have no maturation inducing activity. These females' coelomic fluids were excluded from the analyses above because of the spontaneous spawning that was observed in female 1 (1994) and a failure of female 1a (1995) to spawn at all. In both cases it is likely that the endocrine status of these individuals was different (see section 3.4.1). No significant maturation inducing activity was present in any of the coelomic fluids from females 7-12 and 7a-12a. Incubation of the oocytes directly in homogenised prostomia of  $0.2 \text{ prostomia.ml}^{-1}$  in 1994 and  $1.0 \text{ prostomia.ml}^{-1}$  in 1995 (Treatment 3) and in TFSW only (Treatment 4) also fails to cause any oocyte maturation in both experiments.

The presence of maturation inducing activity in coelomic fluid from females 2-6 and 2a-6a was confirmed by the fertilization of 50% of the oocytes after their incubation in the coelomic fluid samples. Figure 3.2 also shows the mean percentage of oocytes fertilized (hatched bars) after incubation in coelomic fluid of females 1-6 (1994) and 1a-6a (1995). Again only in coelomic fluid from females 2-6 and 2a-6a

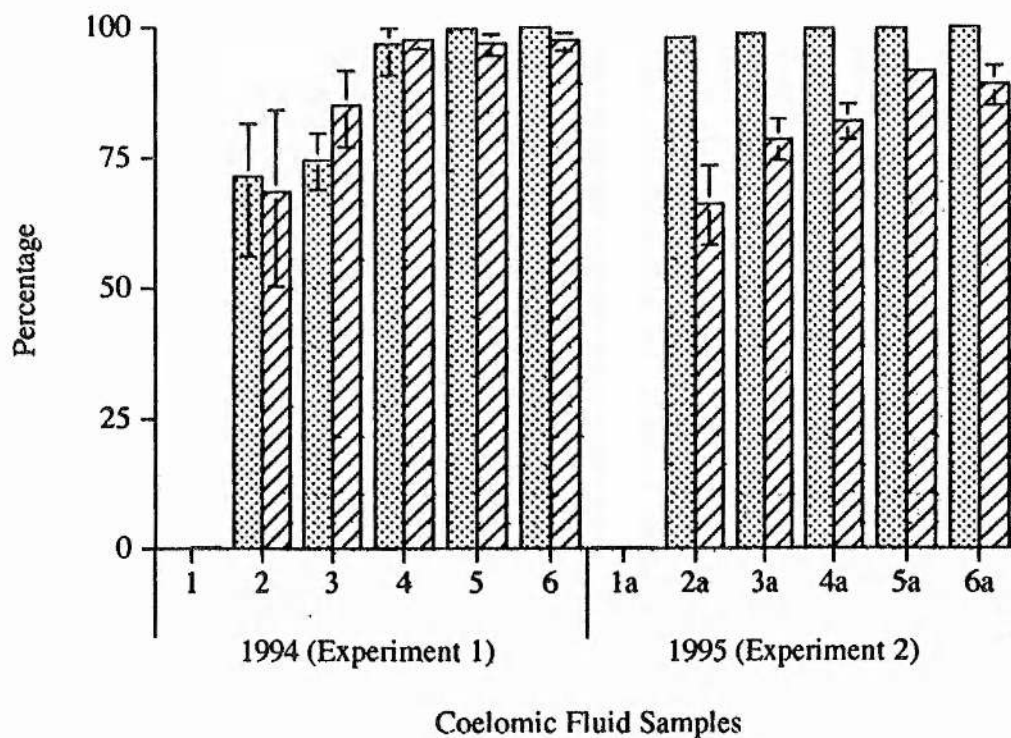


Fig. 3.2. Response of *Arenicola marina* oocytes of oocyte donors to incubation in coelomic fluid from 12 females (1-6, Experiment 1, 1994; 7-12, Experiment 2, 1995) removed 90 minutes after spawning had been initiated in each female by injection of 1 homogenised prostomium equivalent from other females (Treatment 1). All incubations were carried out at 10°C for 3 hours. Response expressed as arcsine back-transformed mean percentage of oocytes ( $\pm$ S.E.M.) matured (stippled bars) and arcsine back-transformed mean percentage of oocytes ( $\pm$ S.E.M.) fertilized (hatched bars). 50 oocytes counted for Experiment 1. Three replicates of 50 oocytes counted from each oocyte donor for Experiment 2. Error bars too small to be discerned are not shown.

(Treatment 1) where the spawning process had been initiated, did oocytes fertilize after incubation in the coelomic fluids. In experiment 2 (1995), significant interaction effects between coelomic fluid samples and oocyte donors used are present ( $F=26.83$ ,  $p<0.001$ ). The inter-individual variation in the ability of oocytes of the six females to fertilize in 1994 and 1995 is also significant; ( $F=3.38$ ,  $p=0.05$ ) and ( $F=46.52$ ,  $p<0.001$ ) respectively. There are also significant differences between the mean percentage of oocytes fertilized after incubation in the different coelomic fluid samples in both 1994 ( $F=7.23$ ,  $p<0.001$ ) and 1995 ( $F=53.00$ ,  $p<0.001$ ).

Maturation inducing activity in coelomic fluid from females 2-6 and 2a-6a was confirmed by fertilising half of the oocytes that were incubated in the coelomic fluid samples. Analysis using the Wilcoxon Signed Rank Test has shown that differences are present between the numbers of oocytes matured and numbers of oocytes fertilized for each coelomic fluid sample. In 1994 these differences are not significantly different ( $T=102.5$ ,  $p>0.05$ ), but in 1995 the number of fertilized oocytes is significantly less from all samples than the number that had matured ( $T=14$ ,  $p<0.001$ ).

### **3.3 The Relationship Between Oocyte Maturation and Spawning**

#### **3.3.1 Materials and Methods**

Gravid *Arenicola marina* were collected and maintained as described in section 3.2. Twenty females were used for each experiment. Ten were each injected with 200  $\mu$ l of homogenate containing 1 prostomium equivalent per female and ten control individuals were injected with 200  $\mu$ l of TFSW as described in section 3.2.1. The experiment was repeated with different individuals on three occasions; 13/10, 19/10 and 26/10 in 1995. Following injection, animals were returned to their containers in 150 ml of TFSW and incubated at 10°C for 8 hours. At 8 hours post-injection, all



individuals' containers were examined for spawned oocytes. Three samples of oocytes were removed and these were assayed for maturation using the fluorescence assay as described in section 2.2.2. Oocytes were also removed from the coelomic cavity of each worm as described in section 3.2.1 and were also assessed for maturation as described 2.2. The procedure was repeated at 24 hours post-injection.

### 3.3.2 Results

The results of the three experiments are summarised in Table 3.2. In the first experiment (13/10/95), all females fail to respond to injection of homogenised prostomia; no mature oocytes in the coelomic fluid or spawned oocytes were present. One week later (19/10/95), mature oocytes are present in the coelomic fluid of only 5 females injected with homogenised prostomia, and all these females subsequently spawned. Two weeks later (26/10/95), as the natural spawning date approached, 9 out of 10 females injected with homogenised prostomia have oocytes that had matured and all 9 of these subsequently spawned. In all three experiments all control females show no evidence of oocyte maturation or spawning.

## 3.4 Discussion

### 3.4.1 The Coelomic Maturation Factor

In 1994, experiment 1 used a prostomial concentration of 0.2 prostomia.ml<sup>-1</sup> for the *in vitro* incubation of oocytes. This was the highest concentration used to induce oocyte maturation *in vitro* by Meijer and Durchon, (1977). In experiment 2, to confirm that the injection of homogenate containing 1 prostomia equivalent per ml to induce the production of the second substance was not acting directly on the oocytes to induce maturation, a concentration of 1 prostomia.ml<sup>-1</sup> was used for incubating oocytes *in vitro*. In both cases no maturation of oocytes occurred. These results confirm work by Auckland (1993) that incubation of oocytes from Scottish

Treatment	Female	13/10/95		19/10/95		26/10/95	
		Mature oocytes	Spawned	Mature oocytes	Spawned	Mature oocytes	Spawned
Females Injected with homogenised prostomia	1	-	-	-	-	+	+
	2	-	-	+	+	+	+
	3	-	-	-	-	-	-
	4	-	-	-	-	+	+
	5	-	-	+	+	+	+
	6	-	-	+	+	+	+
	7	-	-	-	-	+	+
	8	-	-	+	+	+	+
	9	-	-	-	-	+	+
	10	-	-	+	+	+	+
Control females	11-20	-	-	-	-	-	-

Table 3.2. Results are from three separate experiments carried out on the 13/10, 19/10 and 26/10 1995 using the same protocol but different *Arenicola marina* females all collected from West Sands, St Andrews. Females (1-10) were each injected with 200 µl of 1 equivalent homogenised prostomia. Control females (11-20) were injected with 200 µl TFSW. All were assayed for presence (+) or absence (-) of mature oocytes within their coelomic fluid at 8 and 24 hours post-injection. All females were also assessed for presence of spawning (+) or absence of spawning (-) at 8 and 24 hours post-injection. All females were maintained at 10°C under ambient illumination. Three samples of 50 oocytes were removed from each females' coelomic fluid to assess for oocyte maturation.

populations of *Arenicola marina* directly in homogenised prostomia fails to induce maturation. In comparison, the coelomic fluid of 10 out of the 12 females injected with homogenised prostomia induced significant levels of maturation in the oocytes. Taking these results together confirms the presence of a second substance found in females' coelomic fluid after injection of homogenised prostomia (all coelomic fluid from control females, injected with TFSW, had no maturation inducing activity). Auckland (1993), suggested that *Arenicola marina* oocytes may have a different mechanism for oocyte maturation to that described earlier for this species (Meijer and Durchon, 1977). It now seems likely that the earlier reports of Meijer and Durchon (1977) on *A. marina* were actually studies on the recently described species *Arenicola defodiens* (Cadman and Nelson-Smith, 1993) carried out on specimens from Northern France. Oocyte maturation in *A. marina* is controlled by a two step process. It involves a substance from the prostomium (prostomial maturation hormone), the action of which results in the appearance in the coelomic fluid of maturation inducing activity; a Coelomic Maturation Factor (CMF). Oocyte maturation described by Meijer and Durchon (1977), probably in *Arenicola defodiens*, is, in contrast, a single step process as oocytes can be matured by incubation directly in homogenised prostomia.

The comparison of the two systems of oocyte maturation of *Arenicola marina* and *A. defodiens* under identical conditions, and experiments of an interspecific nature are of a high priority. However, these experiments could not be performed because of severe time constraints during the reproductive season and because the reproductive seasons of the two species do not overlap.

The production of CMF can be induced routinely by injecting gravid females with homogenised prostomia causing the initiation of the spawning process. Other workers have noted that as the natural spawning date approaches there is a tendency for females to spawn spontaneously in the laboratory (Howie 1963, 1984; Farke and Berghuis, 1979). In 1994, (experiment 1) of section 3.3.2, one of the females from which coelomic fluid was harvested spawned spontaneously and the resulting

coelomic fluid that was assayed had lost CMF activity. In 1995 (experiment 2), coelomic fluid harvested from one of the females also had no CMF activity, but had failed to spawn spontaneously. It is more likely that this coelomic fluid had been harvested too early for the production of CMF. The timing of CMF production after injection of homogenised prostomia is discussed in detail in Chapter 4.

The assumption that a mature oocyte can always be fertilized has been shown in these experiments to be incorrect. In 1995, although not in 1994, the number of fertilized oocytes was significantly lower than the number of oocytes that matured after incubation in active coelomic fluid. This suggests that either there was a problem with the sperm or that oocytes, although capable of maturing, are not always capable of being fertilized. This justifies using the fluorescence assay rather than the fertilization of oocytes to assess maturation levels and therefore the levels of CMF activity or other maturation inducing substances present.

### **3.4.2 The Relationship between Oocyte Maturation and Spawning**

The injection of prostomial homogenate into females prior to the breeding season (Howie, 1966) and sometimes during the breeding season (pers. obser.) does not always induce spawning. Howie (1961b) proposed that a change in shape of the oocyte during maturation resulted in the automatic acceptance of these oocytes into the nephromixia and subsequent spawning. Results described in 3.3.2 show that as the natural spawning date approaches (which in 1995 at W. Sands, St Andrews occurred between the 5th and 8th of November) the number of females responding to injection of prostomial homogenate increases. In all females, where mature oocytes were present within their coelomic fluid, spawning occurred within 24 hours after injection. In females where no oocytes matured, no spawning took place. The experiments demonstrate that when the maturation system is initiated by injection of homogenised prostomia and oocytes mature, then these oocytes will always be spawned. A failure to spawn (after injection of prostomial homogenate) in females prior to, or during the

breeding season, is not due to the inability of the nephromixia to accept mature oocytes, but because the oocytes never mature. This is because of a failure in the maturation process as a whole or one of its intermediate steps. The experiments confirm that spawning is dependent upon oocyte maturation and once mature, oocytes are always spawned. Spawning within the breeding season is more than likely to be an automatic response to oocyte maturation as proposed by Howie (1961b) due to the morphological changes occurring at maturation to oocytes.

## **Chapter 4**

### **The Production and Action of the Coelomic Maturation Factor (CMF) in *Arenicola marina***



## 4.1 Introduction

In *Arenicola marina* the prostomium maturation substance is only present during the breeding season (Howie, 1966). The equivalent hormone to this substance in starfish is the Gonad Stimulating Substance (GSS). This substance is present in the radial nerves throughout the year, irrespective of the breeding season (Chaet, 1966). However, GSS is found in the coelomic fluid only during the breeding season (Kanatani and Ohguri, 1966; Kanatani and Shirai, 1970). GSS induces maturation through a second substance, 1-methyladenine (1-MeAde) (Kanatani 1969; Kanatani *et al.*, 1969; Kanatani and Shirai, 1970; Hirai *et al.*, 1973), which is equivalent in function to CMF. Immediately after the application of GSS, 1-MeAde is produced *de novo*; it is not a breakdown product or the result of activating a precursor (Shirai, 1972).

In this chapter the production of CMF in *Arenicola marina* is explored in relation to two areas. From Chapter 3 it is known that the injection of homogenised prostomia induces CMF production. However, the precise timing of production after injection is not known and this is examined in individual females in section 4.2. The production of CMF may also be related at a population level to the approach of the spawning period. Section 4.3 examines CMF production on a population level to see if any increases in protein content of the coelomic fluid of females in a population, as the spawning season approaches, can be related to a build up of inactive CMF (or a precursor) in the coelomic fluid.

The incubation times required for 1-MeAde to induce maturation in starfish oocytes (the hormone dependent period [HDP]) and threshold concentrations at which it induces oocyte maturation are well documented (see Chapter 1). Data presented in section 4.4 describes the action of CMF in relation to incubation times required for it to induce maturation. Section 4.5 examines the effects of dilution on CMF activity

and the possible presence of a threshold concentration below which oocyte maturation fails to occur.

## **4.2 The Timing of Production of CMF after Induction of Spawning**

### **4.2.1 Materials and Methods**

For all the following sections in this chapter except 4.5, *Arenicola marina* were collected, maintained and assessed for maturity as described in section 3.2.1. For section 4.5 collection and maintenance was as described in that section's materials and methods. In all the following sections, the assessment of maturation using either unfixed or fixed oocytes and subsequent staining protocol was dependent on the time constraints for each experiment only.

#### **Experimental Design**

This experiment was carried out in November 1994 with animals collected from the Eden Estuary and in November 1995 with animals from West Sands. The experiment in 1995 was a repeat of 1994 except that 10 females were injected and assayed for CMF activity as opposed to 5 in 1994. Three females on both occasions were used to provide oocytes for the assay of maturation inducing (CMF) activity as described in section 4.2.1.

Prior to the injection of 200  $\mu$ l of homogenate containing 1 prostomium equivalent to induce spawning, each female had 150  $\mu$ l of coelomic fluid removed as described in section 3.2.1. Once injection of prostomial homogenate was complete, further 150  $\mu$ l samples of coelomic fluid were removed from each female at the following times post-injection: 0.5, 1, 2, 3, 4, and 5 hours. All coelomic fluid samples removed at these times and the original coelomic fluid sample removed prior

to injection, from each female, were assessed for CMF activity with oocytes from the 3 oocyte donors, which were then fixed after incubation as described in section 2.2.2.

On each occasion that coelomic fluid was harvested from the females, a sample of the females own oocytes removed within the coelomic fluid were fixed immediately and then assessed for their maturational state using the fluorescence assay as described in section 2.2.2.

### **Statistical Analysis**

The complexity of modelling data over time including interaction effects and the serious violation of assumptions of usable models resulted in confining the analysis to the visual inspection of graphically presented data.

### **4.2.2 Results**

The results of experiments conducted in 1994 and 1995 (Figs. 4.1 a-e and 4.2 f-l respectively) on the timing of CMF production in individual females after injection of homogenised prostomia show that inter-individual variation in the timing and amounts of CMF produced (percentage number of oocytes maturing) is large. Comparisons can be made between individuals from within the same experiment because the oocyte donors used within that experiment are the same. However, direct comparisons between individuals from 1994 (a-e) and 1995 (f-l) experiments would be invalid because the oocyte donors are different.

In the experiment conducted in 1994, 5 females (a-e) were injected and all coelomic fluids from each female have detectable CMF levels (Fig. 4.1). In 1995 out of 10 females injected 7, (f-l), have detectable levels of CMF (Fig. 4.2). The three that failed to produce CMF were discarded. The wide variation between individuals' responses warrants that each individual be discussed in turn. However, individuals within each experiment can be grouped both in terms of similar production times and similar amounts of CMF produced. In all females tested, from both experiments, no

Fig. 4.1. Mean response (stippled bars) of *Arenicola marina* oocytes from 3 donors to incubation in coelomic fluid samples removed from 5 females (a-e) prior to the initiation of spawning at time 0, and at 0.5, 1, 2, 3, 4 and 5 hours after spawning had been initiated in each female by injection of 1 equivalent homogenised prostomium. The number of each female's (a-e) own oocytes showing maturation (red line). These oocytes were removed in conjunction with coelomic fluid removal at the same times as coelomic fluid samples. This experiment was performed in 1994, all incubations were carried out at 10°C for 3 hours and oocytes to be assessed were fixed immediately. Responses expressed as arcsine back-transformed mean percentage ( $\pm$ S.E.M.) of oocytes matured. Three replicates of 50 oocytes counted from each oocyte donor and females' own oocytes removed with coelomic fluid.



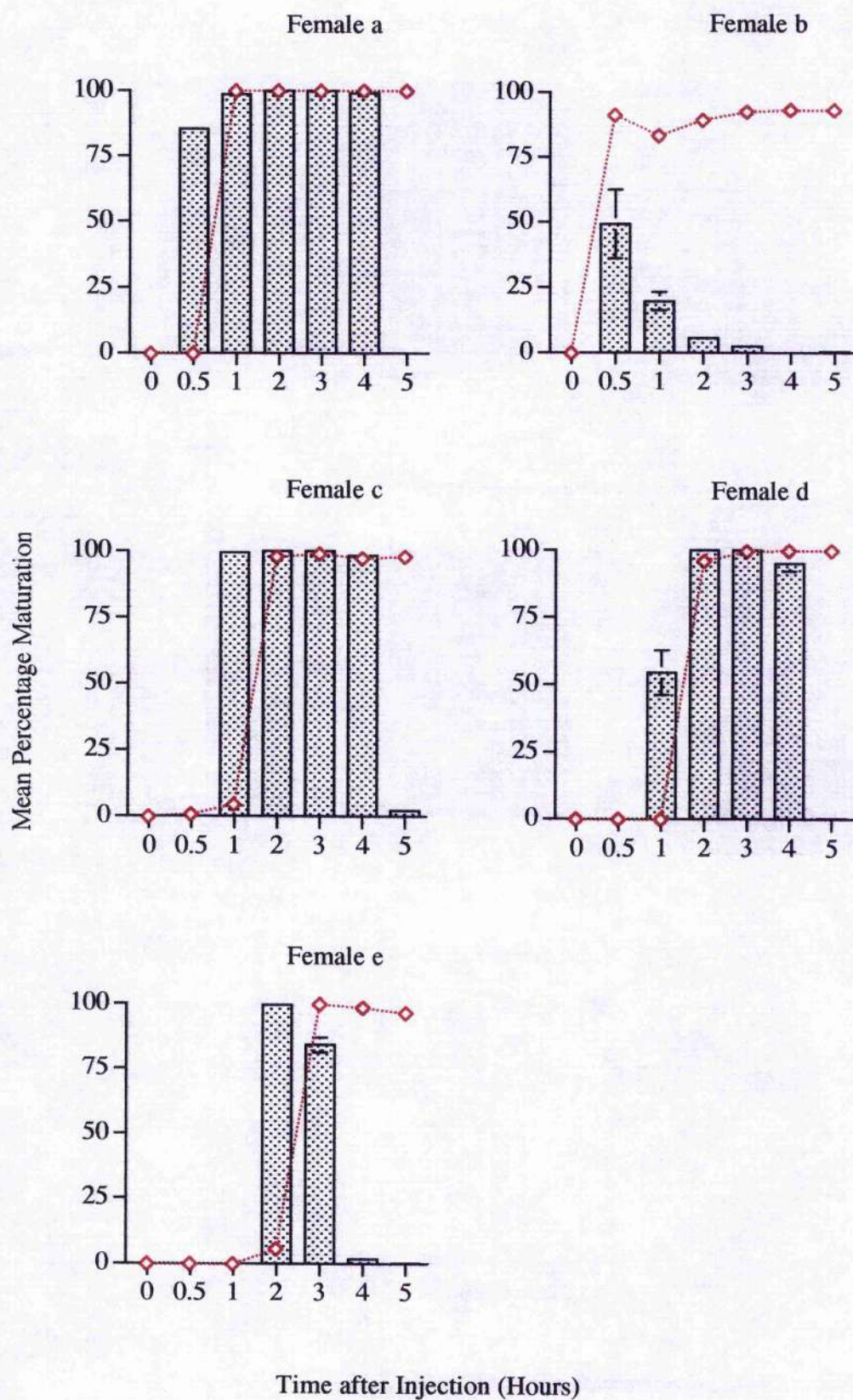
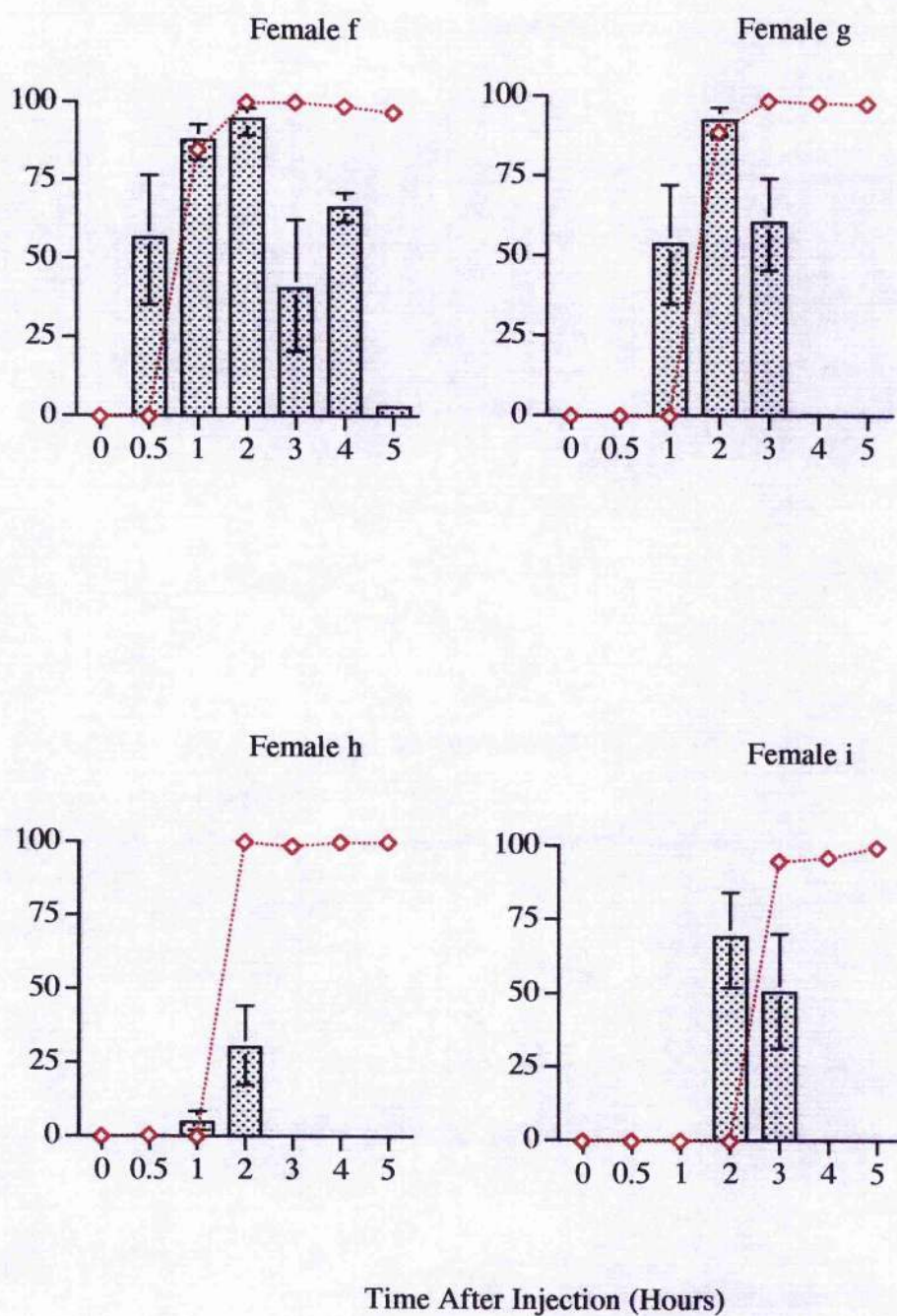


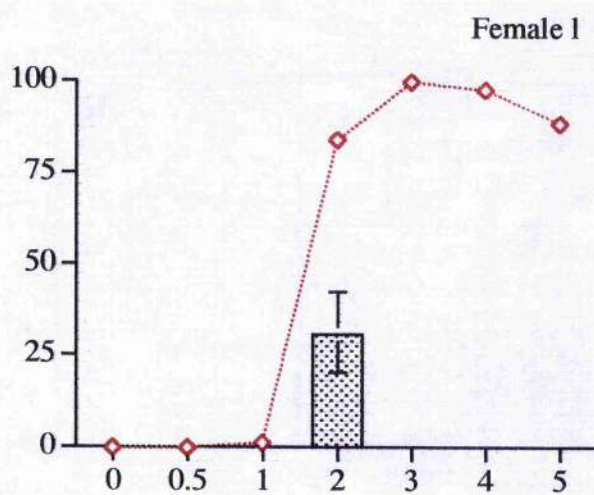
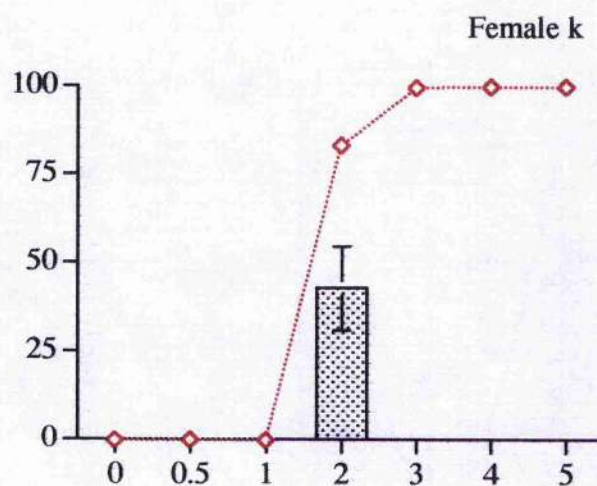
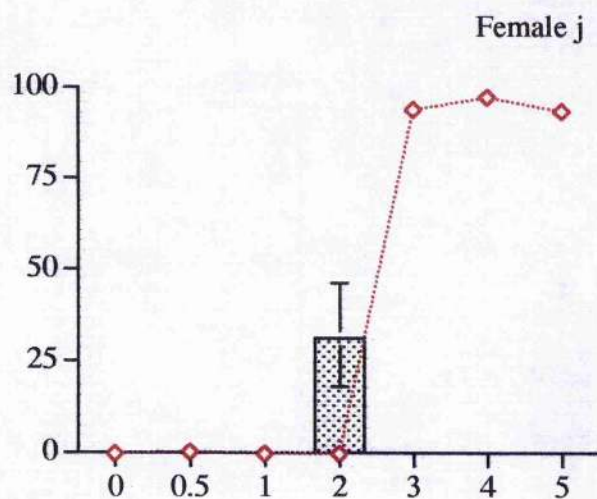
Fig. 4.2. Mean response (stippled bars) of *Arenicola marina* oocytes from 3 donors to incubation in coelomic fluid samples removed from 7 females (f-l) prior to the initiation of spawning at time 0, and at 0.5, 1, 2, 3, 4 and 5 hours after spawning has been initiated in each female by injection of 1 equivalent homogenised prostomium. The number of each female's (f-l) own oocytes showing maturation (red line). These oocytes were removed in conjunction with coelomic fluid removal at the same times as coelomic fluid samples. This experiment was performed in 1995, all incubations were carried out at 10°C for 3 hours and oocytes to be assessed were fixed immediately. Responses expressed as arcsine back-transformed mean percentage ( $\pm$ S.E.M.) of oocytes matured. Three replicates of 50 oocytes counted from each oocyte donor and females' own oocytes removed with coelomic fluid.



Mesal Percentage Maturation



Mean Percentage Maturation



Time After Injection (hours)



CMF was present immediately prior to injection of prostomia (0 hours). Spontaneous maturation of oocytes from oocyte donors is also absent as none matured in the sea water controls (not shown in figures).

In Figure 4.1, both females a and b have detectable CMF levels at 0.5 hours post-injection. In female a, a high level of CMF (100% approx.) is maintained for 4 hours post-injection until it disappears at 5 hours. Female b, in contrast, produces only half this amount of CMF at 0.5 hours post-injection and then subsequently CMF steadily fell until at 4 hours no CMF was present.

In female c, CMF (nearly 100%) is first detected at 1 hour post-injection. This level is maintained until 4 hours post-injection before disappearing at 5 hours. The first detectable levels of CMF in female d are also present at 1 hour post-injection but at only half the level of female c. After this, however, CMF levels are maintained at similar levels to female c before again disappearing at 5 hours. The production of CMF in female e is first detectable after 2 hours post-injection. The amount of CMF is again comparable to females a, c, d and e, but disappeared after 4 hours post-injection.

As with individuals of 1994, individuals from 1995 can be grouped according to the timing and amount of CMF activity produced. In Figure 4.2 only in female f is CMF present at 0.5 hours post-injection. Levels at this time are approximately 60% and then increased to nearly 100% at 1 hour post-injection. There is a sharp drop at 3 hours to 40% followed by a recovery at 4 hours to 60% before nearly a complete disappearance at 5 hours. In comparison to the other six females in 1995, female f has the fastest and greatest production of CMF.

Both females g and h have detectable CMF levels at 1 hour post-injection, although female h has a very low level (10%) in comparison to female g (50%). A high level (100% approximately) of CMF activity in female g is present at 2 hours post-injection but this fell to 60% at 3 hours and disappeared at 4 hours post-injection.

In contrast, levels in female h peaks at 2 hours post-injection (30%) and then disappears at only 3 hours.

CMF activity in coelomic fluid of females i, j, k and l appears 2 hours post-injection. Activity in all these except female i disappears at 3 hours post-injection. In females j, k and l levels of CMF are also very low.

The maturity of each female's own oocytes removed in conjunction with its coelomic fluid sample was also assessed (red line in Figs. 4.1 and 4.2). Across all females two patterns emerge. The first, found in females a, c, d, e, f, g, h, i and j shows that at the time CMF levels became detectable, shown by the *in vitro* induction of maturation of oocyte donor's oocytes, the individuals' own oocytes removed at the same time as the coelomic fluid have not matured. The situation found in females b, l and k was that at the time CMF becomes detectable some of the individuals' own oocytes have also matured.

Figures 4.3 and 4.4 are summary figures of the grand means of all 12 individuals for levels of CMF and maturation of individuals own oocytes respectively at times post-injection. Figure 4.4 shows that at 0.5 hours post-injection CMF has induced maturation in 10% of the oocytes but this increased to 25% after 1 hour. The peak levels of CMF are reached at 2 hours post-injection, when 75% of the oocytes are induced to mature, after which the numbers of mature oocytes falls off to a complete disappearance at five hours.

Figure 4.4 shows that at 0.5 hours post-injection very few of an injected individual's own oocytes removed with the coelomic fluid have matured. At 1 hour post-injection more CMF is detectable (shown in Fig. 4.3) but still few of these oocytes are mature. This corresponds to the time when although CMF levels are detectable, exposure to CMF of the females own oocytes has not been long enough to induce maturation in these oocytes. At 2 hours the majority of oocytes removed are

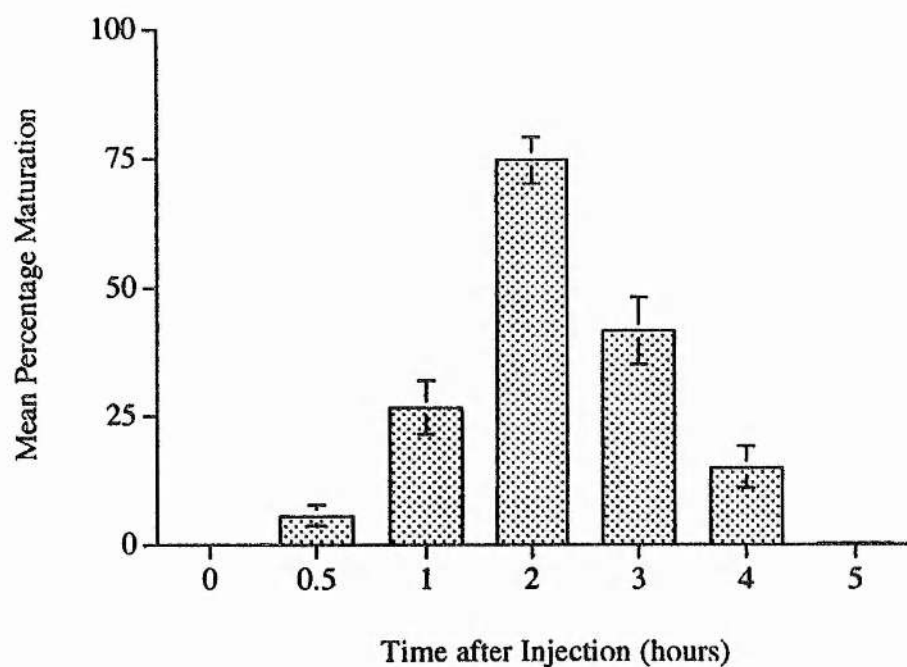


Fig. 4.3. Mean percentage number of matured oocytes of all oocyte donors to incubation in coelomic fluid samples removed from all females (a-l) prior to the initiation of spawning at time 0, and at 0.5, 1, 2, 3, 4 and 5 hours after spawning had been initiated in each female by injection of 1 homogenised prostomium equivalent. All incubations were carried out at 10°C for 3 hours and oocytes to be assessed were fixed immediately. Response expressed as arcsine back-transformed mean percentage ( $\pm$ S.E.M.) of oocytes matured. Three replicates of 50 oocytes counted from each oocyte donor.

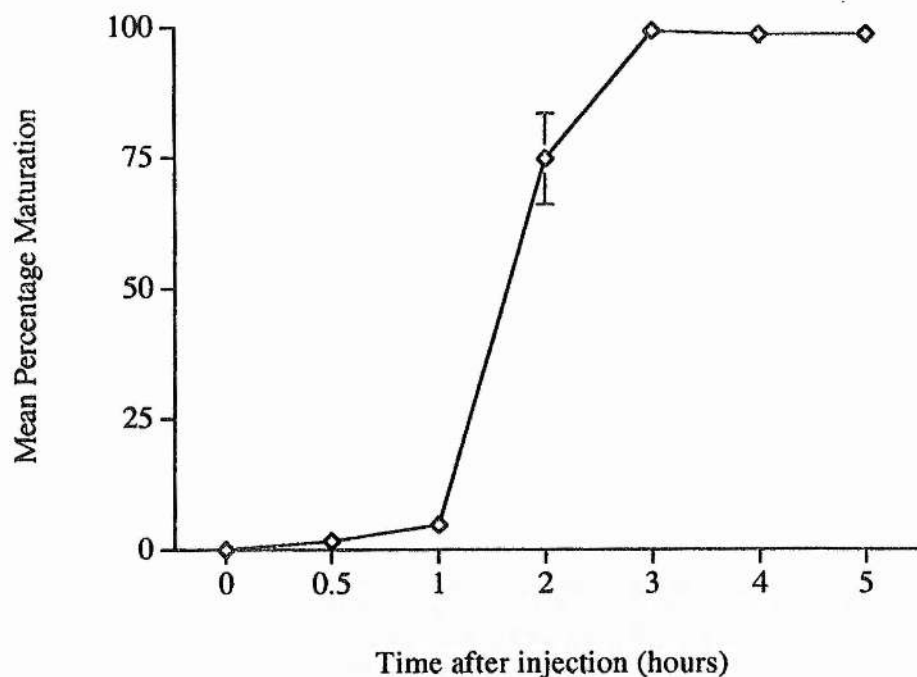


Fig. 4.4. Mean percentage number of all females' (a-l) own oocytes matured, removed in conjunction with coelomic fluid removal at the same times (0 and at 0.5, 1, 2, 3, 4, and 5 hours after spawning had been initiated) as coelomic fluid samples and assessed for maturation. All incubations were carried out at 10°C for 3 hours and oocytes to be assessed were fixed immediately. Response expressed as arcsine back-transformed mean percentage ( $\pm$ S.E.M.) of oocytes matured. Three replicates of 50 oocytes counted from females' own oocytes removed with coelomic fluid. Error bars too small to be discerned are not shown.



mature, and by 3 hours nearly 100% of the oocytes are mature. This level is then maintained through 4 and 5 hours post-injection.

## **4.3 Monthly Determination of Protein Content of Coelomic Fluid**

### **4.3.1 Materials and Methods**

#### **Collection and Maintenance of Animals**

The collection of *Arenicola marina* for this experiment required a natural population of animals of high density and easy access which precluded all other sampling sites except the Eden Estuary population. Animals were collected (as described in section 3.2.1) on the 22nd of each month or as near to this date as the tidal heights permitted for 12 months (Jan. to Dec.) during 1995. Animals were maintained in running sea water tanks at ambient temperature with a small amount of sediment for a minimum of 24 hours (to allow the evacuation of their gut contents) under ambient illumination.

Approximately 80 adults and 30 juveniles were collected per month. This was found to be sufficient numbers for the 15 males, 15 females and 15 juveniles to be tested. Juveniles were collected from the juvenile beds at the top of the beach. Adults were collected from the adult population in the middle to lower area of the beach. Juveniles were also classed by the lack of developing gametes, although in May and June samples, the juveniles collected did have gametes present (see Discussion).

#### **Removal of Coelomic Fluid from Animals**

A sample of 140  $\mu$ l of coelomic fluid (adequate for both standard and micro assays) was removed from each worm as described in section 3.2.1. Individual samples were discarded if any blood was evident in the removed coelomic fluid. All

removed coelomic fluid was stored in Eppendorf tubes on ice until 15 samples of male, 15 samples of female and 15 samples of juvenile coelomic fluid were collected each month.

### **Analysis of Protein Concentration of Coelomic Fluid**

Standard curves were produced for each assay with albumin standards, made up of fraction V bovine serum albumin in 0.9% NaCl with sodium azide (Pierce) with a protein concentration of 2 mg.ml<sup>-1</sup>. This was diluted in TFSW to obtain the standard curve concentrations required.

Protein concentration analysis of the coelomic fluid samples and standards was carried out using the Coomassie® Protein Assay Reagent Kit (Pierce) based on the Bradford method (1976). Two replicate samples of each individual's coelomic fluid were assayed against the blank of the standard curve. Absorbance was measured at 595 nm using an LKB Biochrom Ultraspec K Spectrophotometer.

### **Assessment of Maturity of Sampled Individuals**

The maturity of males was assessed by estimating the number of spermatocyte clusters and morulae under a compound light microscope using a x10 objective from 3 fields of view. The mean percentage of sperm present as morulae was then calculated. The diameters of 50 oocytes taken from females were measured using a graduated eye piece, calibrated with a micrometer slide.

### **Statistical Analysis**

The complexity of modelling data over time including interaction effects and serious violation of model assumptions resulted in confining analysis to visual inspection of graphically presented data and the use of regression plots for correlations.

### 4.3.2 Results

The mean protein concentration for each month for juveniles, females and males are shown in Figure 4.5a-c respectively. Mean juvenile protein concentration remain consistently low ( $150\text{--}350\ \mu\text{g.ml}^{-1}$ ) throughout the twelve month period except for a small increase in January to  $450\ \mu\text{g.ml}^{-1}$  and a very large increase in June to  $900\ \mu\text{g.ml}^{-1}$ . The high value in June consists of juveniles with large inter-individual variation as shown by the large standard errors. In Figure 4.5b mean female protein concentration also remains consistently low from January to September ( $250\ \mu\text{g.ml}^{-1}$  approximately) with very low levels being attained in March ( $120\ \mu\text{g.ml}^{-1}$ ) and September ( $150\ \mu\text{g.ml}^{-1}$ ). However, in October, prior to the majority of the natural spawning in November, there is an increase in mean protein concentration to nearly  $500\ \mu\text{g.ml}^{-1}$  which then tails off rapidly in November and December. Mean protein concentration of males also follows a similar pattern to that of juveniles and females up until May; with relatively low concentrations of around  $300\ \mu\text{g.ml}^{-1}$  and a drop in March to  $130\ \mu\text{g.ml}^{-1}$ . An increase in June to  $500\ \mu\text{g.ml}^{-1}$  is followed by a further increase in July to  $700\ \mu\text{g.ml}^{-1}$  and then a decrease in August and a further reduction in September to  $150\ \mu\text{g.ml}^{-1}$ . A large increase in October to  $800\ \mu\text{g.ml}^{-1}$  is followed by levels similar to females and juveniles in November and December. In months where there are increases in the mean protein concentration there are concomitant increases in the variation within samples for that month shown by the large standard errors.

The mean oocyte diameter and mean percentage number of morula for each female and male are presented in Figures 4.6 and 4.7 respectively. In females from December to April, only oocytes at very early stages of development are present. From May onwards there is a continued increase in mean oocyte diameter peaking in July and then dropping off in both August and a further reduction in September. An increase in October is followed by a large fall in November.

Fig. 4.5. Mean protein concentration of coelomic fluid ( $\mu\text{g.ml}^{-1} \pm \text{S.E.M.}$ ) from 3 classes of *Arenicola marina*: a) juveniles; b) females and c) males. Two replicate samples assayed per individual, fifteen individuals per class per month. All individuals removed from the Eden Estuary on the 22nd of each month or as near to this date as possible for twelve months (January to December). Error bars too small to be discerned are not shown.

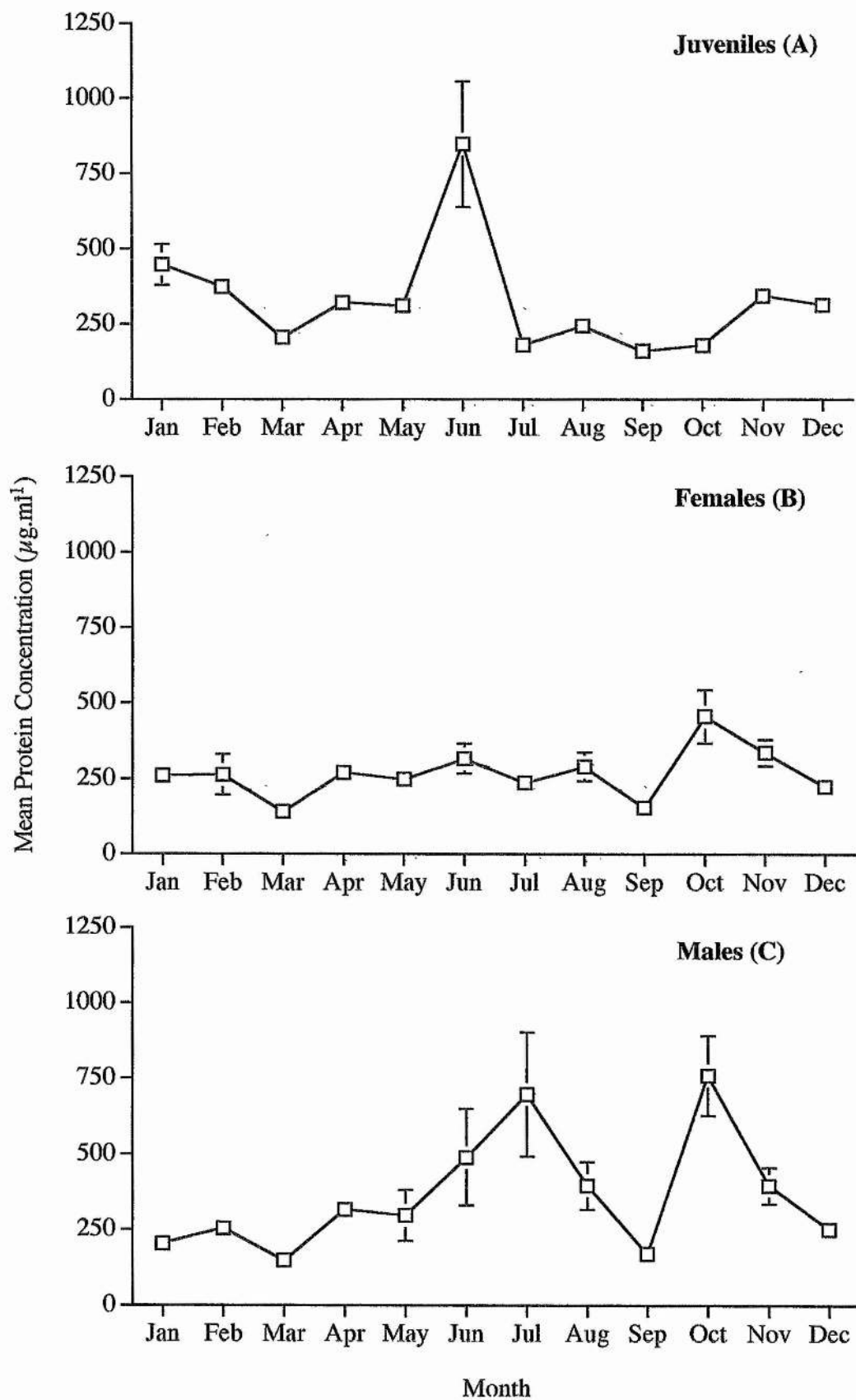
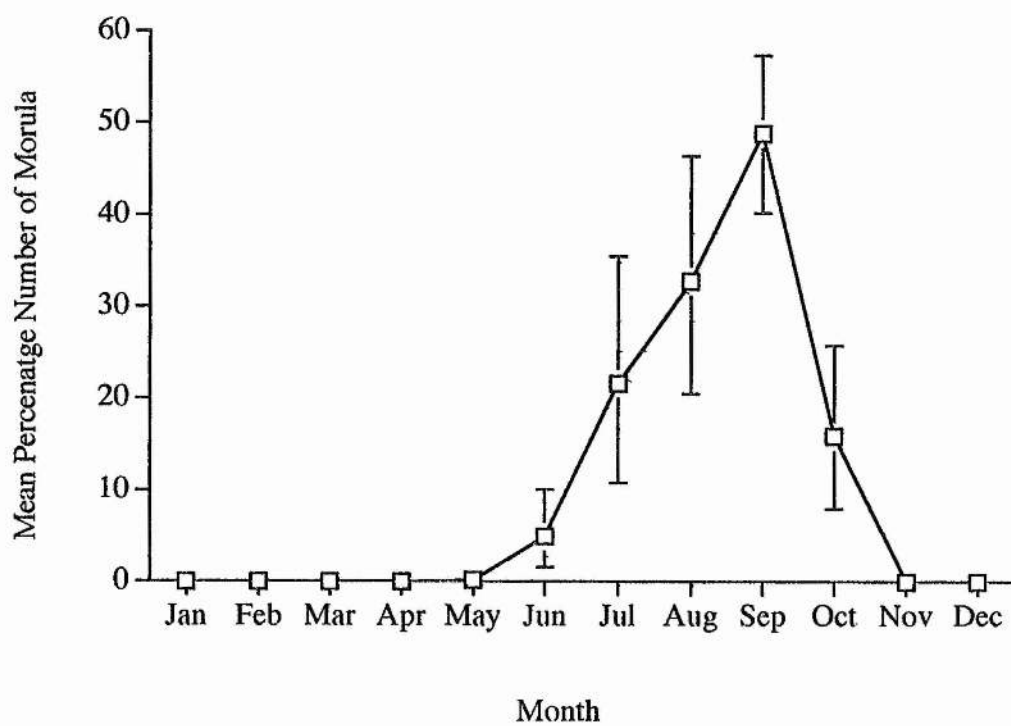
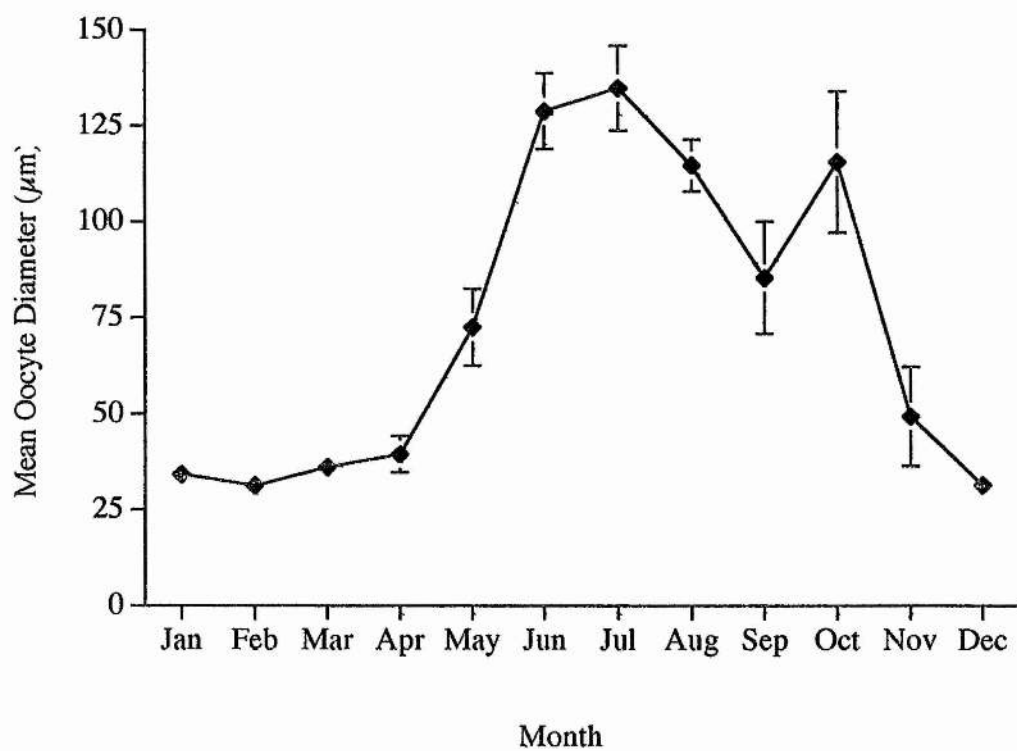


Fig. 4.6. Mean oocyte diameter ( $\mu\text{m} \pm \text{S.E.M.}$ ) of female *Arenicola marina* which were assayed for protein concentrations. Fifty oocytes counted per female, fifteen females per month for 12 months (January to December). All individuals removed from the Eden Estuary on the 22nd of each month or as near to this date as possible. Error bars too small to be discerned are not shown.

Fig. 4.7. Mean percentage number of morula ( $\pm \text{S.E.M.}$ ) in male *Arenicola marina* which were assayed for protein concentrations. Numbers of morulae were estimated from 3 fields of view. The mean percentage of sperm present as morulae was then calculated per male, fifteen males per month for 12 months (January to December). All individuals removed from the Eden Estuary on the 22nd of each month or as near to this date as possible. Error bars too small to be discerned are not shown.





A similar pattern exists for the mean percentage number of morula in males. In November, immediately after the natural spawning time, none of the sampled worms have morula present as they are spent and this state was maintained until April. From May onwards there is an increase in the mean percentage number of morula until a peak of 50% is reached in September. A drop in October indicates some of the sampled worms had already begun to spawn.

A correlation between mean protein concentration of individual males and their mean number of percentage morula is presented in Figure 4.8. The high level of scatter accounts for the weak positive correlation between mean percentage morula and mean protein concentration for individual males over all months ( $R^2=0.241$ ). In Figure 4.9 mean protein concentration of individual females and their mean oocyte diameters are also correlated. The very high levels of scatter accounts for the very weak positive correlation between mean protein concentration and mean oocyte diameter for individual females over all months ( $R^2=0.049$ ).

## **4.4 Incubation Time Required for CMF to Induce Oocyte Maturation**

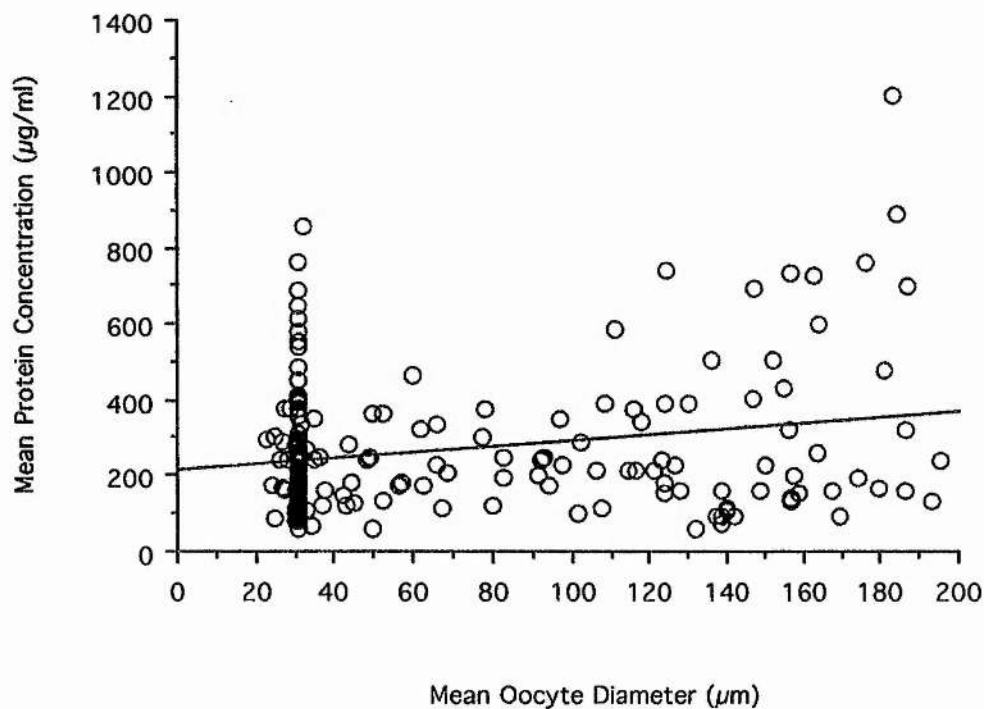
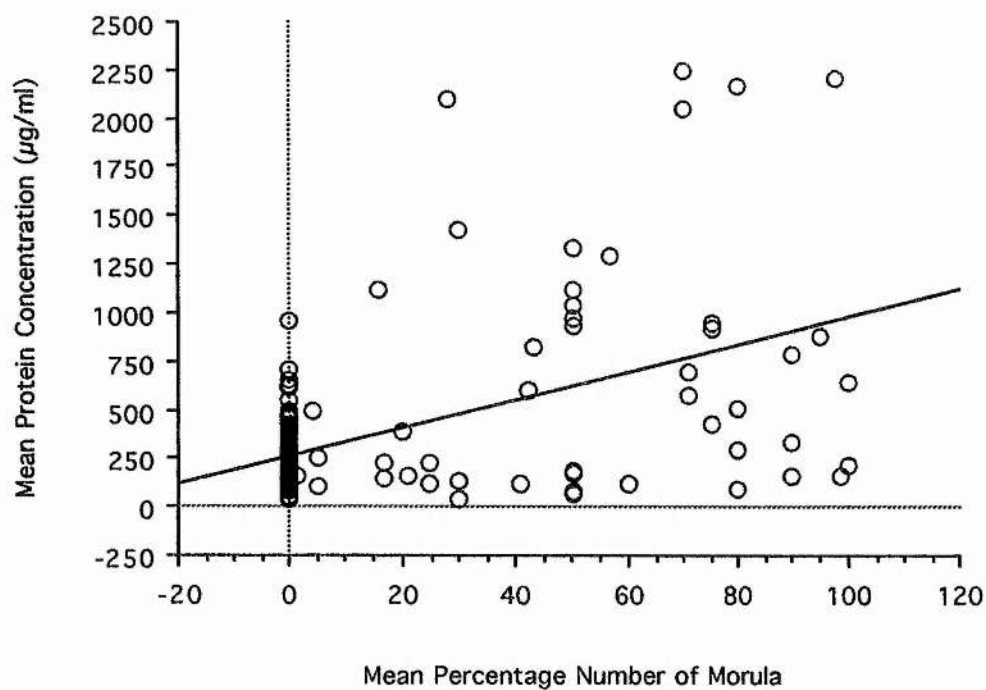
### **4.4.1 Materials and Methods**

#### **Experimental Design**

Nine treatment groups, as shown in Table 4.1, were used to determine the incubation time required for CMF to induce oocyte maturation. In each, 5  $\mu$ l of oocytes from each of 5 oocyte donors were incubated in 50  $\mu$ l of the active coelomic fluid. This was harvested from 4 females and pooled as described in section 3.2. After incubation at the times shown in Table 4.1 at 10°C under ambient illumination conditions, oocytes were removed and washed 3 times in TFSW. Oocytes were then incubated for 3 hours in TFSW to complete any maturational processes that had been

Fig. 4.7. Correlation between mean percentage number of morula and protein concentration of coelomic fluid ( $\mu\text{g.ml}^{-1}$ ) for all *Arenicola marina* males assayed in all months ( $R^2=0.241$ ).

Fig. 4.8. Correlation between mean oocyte diameter ( $\mu\text{m}$ ) and mean protein concentration of coelomic fluid ( $\mu\text{g.ml}^{-1}$ ) for all *Arenicola marina* females assayed in all months ( $R^2=0.049$ ).



initiated. Oocytes from these treatments and the positive and negative control were then assayed using the fluorescence assay as described in section 2.2.2.

Treatment	Time oocytes incubated in CMF (minutes)
1	5
2	10
3	20
4	30
5	40
6	50
7	60
8	80
9	100

Table 4.1. Incubation times of oocytes in CMF relevant to treatment number as referred to in the text.

### *Controls*

Oocytes were incubated in coelomic fluid with CMF activity for 3 hours at 10°C under ambient illumination without washes for a positive control, and also TFSW for 3 hours at 10°C under natural illumination without washes for a negative control.

### **Statistical Analysis**

Statistical analysis was carried out using generalised linear interactive modelling (GLIM Version 3.77). The inclusion of interaction effects resulted in overparametisation within the model and consequently interaction effects were ignored.

#### **4.4.2 Results**

Oocytes from oocyte donors were exposed to coelomic fluid with CMF activity for different times and the results are shown in Figure 4.9. Within the experiment there is significant inter-individual variation in the maturational ability of oocytes from different oocyte donors ( $F=6.14$ ,  $p<0.001$ ). The differences between all treatments are also highly significant ( $F=60.3$ ,  $p<0.001$ ). Oocytes incubated in sea water, as a control, failed to mature and no significant oocyte maturation occurred in oocytes exposed to CMF for 5 minutes in comparison to the sea water control ( $T=1.204$ ,  $p>0.05$ ). An exposure time of 10 minutes to CMF results in significant levels of oocyte maturation in comparison to the control ( $T=2.55$ ,  $p=0.05$ ), but this level (40%) is significantly lower than maturation levels after an incubation time in CMF of 20 minutes ( $T=9.18$ ,  $p<0.001$ ). An incubation time of 20 minutes, when compared to all other incubation times greater than 20 minutes, does not result in significantly different maturation levels ( $F=2.82$ ,  $p>0.05$ ).

### **4.5 The Effect of Dilution on CMF Activity**

#### **4.5.1 Materials and Methods**

##### **Experimental Design**

Coelomic fluid with CMF activity (harvested from 4 females as described in section 3.2.1) was diluted with TFSW into 9 treatment groups, as shown in Table 4.2. Each dilution was divided into six 50  $\mu$ l samples and assayed for CMF activity as described in section 3.2.1 using oocytes from 6 oocyte donors, collected as described in section 3.2.1.



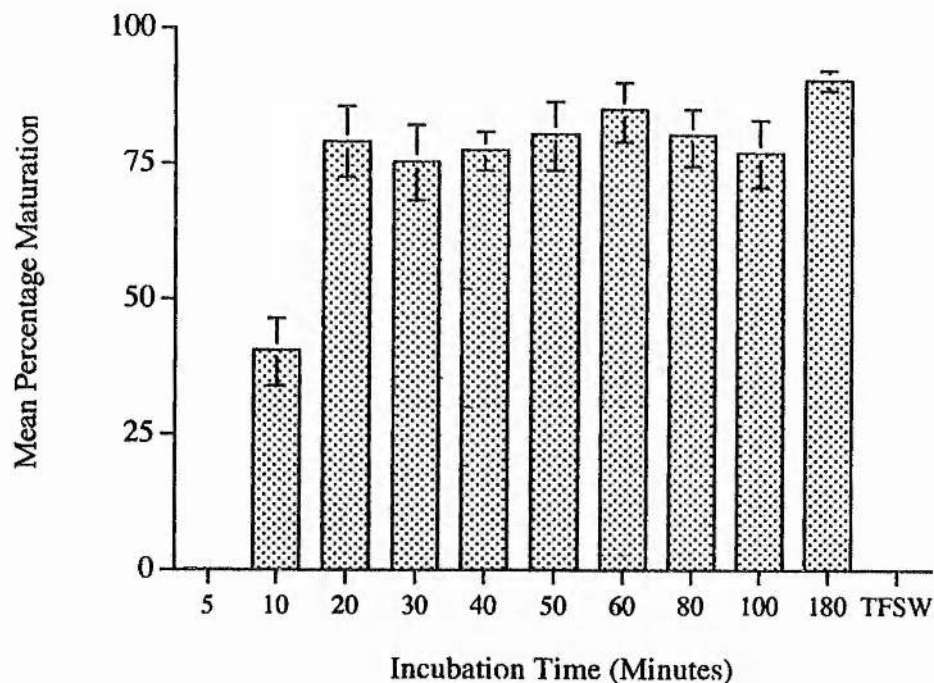


Fig. 4.9. Response of *Arenicola marina* oocytes of 6 oocyte donors to incubation in coelomic fluid with CMF activity for different times and in TFSW only as a control, (incubated for 3 hours). All oocyte incubations in coelomic fluid were carried out at 10°C for 5, 10, 20, 30, 40, 50, 60, 80, 100 and 180 minutes followed by washing and then incubation in in TFSW for 3 hours. Response expressed as arcsine back-transformed mean percentage ( $\pm$ S.E.M.) of oocytes matured. Three replicates of 50 oocytes counted from each oocyte donor.

Treatment	Coelomic Fluid ( $\mu$ l)	TFSW ( $\mu$ l)	% Coelomic Fluid
1	300	0	100
2	240	60	80
3	180	120	60
4	120	180	40
5	60	240	20
6	30	270	10
7	15	285	5
8	3	297	1
9	0	300	0

Table 4.2. Relative contributions of coelomic fluid and TFSW ( $\mu$ l) to obtain dilution series of CMF activity relevant to treatment number as referred to in text.

### Statistical Analysis

The nature of the data enabled statistical analysis to be carried out using a two way analysis of variance on arcsine transformed data using the statistical package Minitab ver. 8.2. A multiple comparison Tukey test (Zar, 1984) was used in breaking down these overall significant differences between treatments by comparing two treatments at a time.

#### 4.5.2 Results

The dilution of coelomic fluid and its effects on levels of CMF activity (percentage number of oocytes maturing) are shown in Figure 4.10. Analysis shows that there are significant differences between all treatments including the sea water control ( $F=672.68$ ,  $p<0.001$ ) and very significant differences between the ability of oocytes of the six females used to undergo induced maturation ( $F=200.32$ ,  $p<0.001$ ). There are also significant interaction effects between treatments and females ( $F=22.21$ ,  $p<0.001$ ). Although there are significant differences between females and

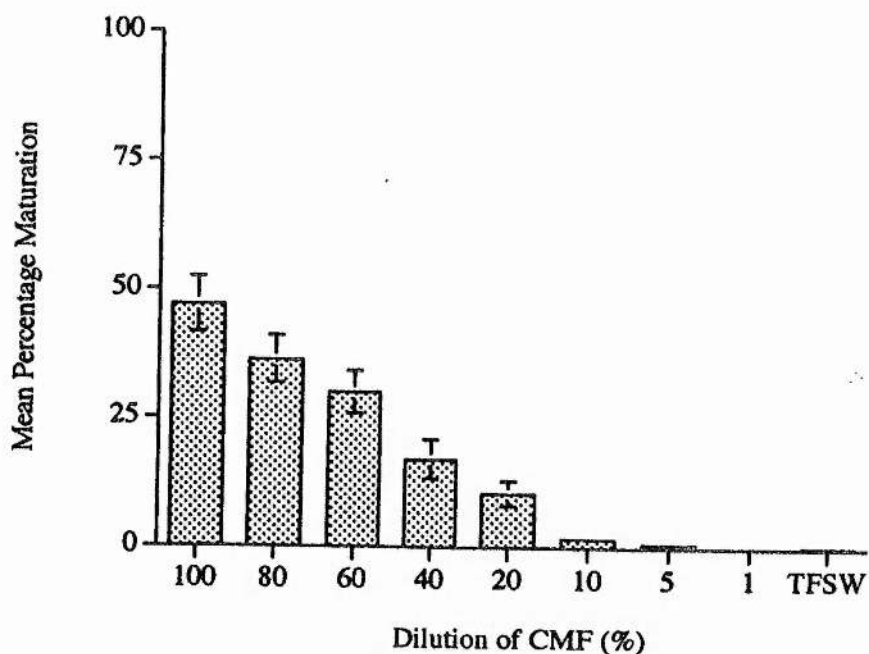


Fig. 4.10. Response of *Arenicola marina* oocytes of 6 oocyte donors to incubation in different percentage concentrations (100, 80, 60, 40, 20, 10, 5, 1) of coelomic fluid containing CMF activity and in a TFSW only (control). All incubations were carried out at 10°C for 3 hours. Response expressed as arcsine back-transformed mean percentage ( $\pm$ S.E.M.) of oocytes matured. Three replicates of 50 oocytes counted from each oocyte donor. Error bars too small to be discerned are not shown.

significant interaction effects, only differences between treatments are important for this experiment. The results of the Tukey (multiple comparison) test show that all treatments are significantly different ( $p=0.05$ ) from each other except for Treatments; 7 and 8, 7 and 9, 8 and 9: There is no significant difference between the SW control and 1% CMF, or between the SW control and 5% CMF, although there is a significant difference between 1% and 5% CMF, but not between the 5% and 10% dilution. All other combinations of treatments are significantly different.

## 4.6 Discussion

The maturational ability of coelomic fluid (amount of CMF present) and the ability of oocytes from oocyte donors to mature have been shown in many of the experiments to be significantly different between females. The differences in levels of CMF in coelomic fluid are likely to be due to a number of factors. The individual nature of the worms such as size differences will influence the amount of CMF produced. CMF levels may also depend on the timing of production of CMF after injection of homogenised prostomia. Results presented in 4.2.2 show that the time of production of CMF, after injection of prostomia varies considerably between individuals from between 0.5 hours and 2 hours post-injection. In many of the experiments in this chapter and chapter 5, all coelomic fluids from the injected females were removed after a pre-determined time, and in the case of the experiment confirming the presence of CMF (section 3.2), removal was at 1.5 hours post-injection. At these specific times CMF production may have been at different levels for each individual so contributing to the significant differences in CMF activity between females.

Inter-individual variation in the ability of oocytes from oocyte donors to mature is likely to be due to the differences in the natural spawning times of individuals and populations and how these relate to the rate at which the oocytes

acquire the ability to mature prior to spawning. During the course of this thesis it has become evident that there is only a small window for a given population in which oocytes from oocyte donors can be matured *in vitro* with CMF. It is likely that this window lies within the slightly longer window when females can be induced to spawn by injection of homogenised prostomia. Both are likely to be directly related to the fact that spawning in natural populations of low synchrony such as the Eden estuary can be spread over a number of weeks and that in the laboratory, worms may be used that spawn at different ends of this natural window. Some of these worms may not therefore be completely mature. This manifests itself in a failure to spawn when injected with homogenised prostomia and so cannot produce CMF, and/or their oocytes do not have the competence to mature in the presence of CMF.

In many of the experiments in this chapter and in chapter 4, interaction effects were present between oocytes from oocyte donors and the different coelomic fluids and treatments used. These interactions are difficult to explain but it may be that different batches of oocytes have different threshold concentrations to CMF above which they mature. Different coelomic fluids will have different levels of CMF so this could give rise to interactions. Small differences in volumes of oocytes and coelomic fluids used may also increase variability and interaction effects. -

Results from 4.2.2 have shown that production of CMF begins between 0.5 and 2 hours after injection of prostomia. In some females this activity is maintained at a high level for up to 4 hours post-injection before being lost at five hours. In other females, activity never reaches a high level and once produced falls off rapidly. These disparities between females may be due to a number of reasons: Damage to the worm after injection often results in blood entering the coelomic fluid (pers. obser.). Work by Clark and Clark, (1962) showed that tissue damage in some polychaetes also results in an increase in the number of coelomocytes in the coelomic fluid. These cells are amoeboid, phagocytic cells which are associated with removal of material from the coelomic fluid. An increase in coelomocytes or their activation by damage (such as

blood in the coelomic fluid) may result in a release of factor(s) or another immune-like response. This may then result, either directly or indirectly, in a rapid breakdown or inactivation of CMF. It is possible that as worms are increasingly damaged due to the sequential sampling, the cellular responses may result in CMF being broken down resulting in a rapid loss of activity.

Another possible reason for loss of CMF is that the production of CMF induces maturation in the females' own oocytes resulting in a shortage for *in vitro* maturation of oocytes. The process by which CMF induces maturation in an oocyte is unknown but may result in CMF being inactivated or broken down after it has induced an oocyte to mature. This may lead to a decrease in active CMF over time as the females' own oocytes mature. This could lead to insufficient CMF being available to induce *in vitro* maturation in oocytes resulting in a rapid fall off in the number of oocytes from oocyte donors maturing.

Results presented in 4.4.2 show that the minimum time oocytes need to be exposed to CMF to mature is 10 minutes, but to get maximum maturation levels comparable with longer incubation times a minimum exposure time of 20 minutes is required. This exposure time of 20 minutes explains results obtained in section 4.2.2 when each female's own oocytes removed in conjunction with its coelomic fluid sample (red line) were also assessed for maturation. Two patterns emerge; the most common pattern (9 out of 12 females), shows that at the time CMF levels become detectable (shown by the induction of maturation in donor female oocytes *in vitro*), the individuals' own oocytes, removed at the same time as the coelomic fluid, had not matured. The other situation found in the remaining 3 females is at the time CMF becomes detectable, the individuals' own oocytes have matured. In the first group, individuals' oocytes may have not been exposed to CMF levels for long enough (20 minutes approximately) to induce maturation before being removed and fixed. Oocytes from oocyte donors incubated *in vitro* in CMF for three hours were exposed for the required time resulting in maturation of these donor oocytes. In the other



situation, at the time CMF is first detected, the individuals' own oocytes have also matured. In these cases, CMF has been produced slightly earlier than in the other females so that the oocytes have had long enough exposure to CMF so have matured before fixing.

The production of CMF in a female after injection of homogenised prostomia has been shown to occur in individual females approximately 2 hours post-injection (see above). Those experiments, however, failed to distinguish whether CMF was produced *de novo* after injection of homogenised prostomia or whether CMF was already present in the coelomic fluid as a precursor or inactive form and injection of homogenised prostomia activated this CMF. Evidence suggests (Chapter 6) that CMF may be a protein so the hypothesis that a protein precursor or inactive form of CMF (termed pre-CMF) accumulates in the coelomic fluid of maturing females as natural spawning approaches was investigated. The measurement of the total protein content of coelomic fluid from 15 females and correlating protein levels to mean oocyte diameter over monthly intervals would examine the accumulation of proteins as spawning approached. In conjunction, a comparison with male and juvenile protein levels would differentiate between the accumulation of proteins due to improvements in nutrition and metabolic changes. In males and juveniles these changes would also be recognised and so these changes could be distinguished from accumulation of proteins for vitellogenesis and pre-CMF in females.

The results presented in Figure 4.5a show that for most of the year the protein concentration of juvenile coelomic fluid is fairly low, although in January there is a small increase. However, in June, mean protein concentration is nearly three times the level in May. This high level of protein in June is in part due to three individuals that have coelomic fluid protein concentrations of nearly 2 mg.ml<sup>-1</sup>, but is also due to a general increase in protein levels in coelomic fluid of most juveniles. A biological explanation for this large increase could be that increased growth rates and improved nutrition through higher sea temperatures may increase the production of proteins in

the coelomic fluid. This, however, is unlikely because of the low protein concentrations in July to October when sea temperatures are higher than June and growth rates and metabolic processes are likely to be the same or greater than in June. Juveniles in both May and June had developing gametes present although they were replaced by juveniles with no gametes in July. This may also be an explanation for the increase in protein concentration in June in juveniles as proteins may be required for gamete development (see below for detailed discussion). In July the replacement of these "pseud juveniles" with juveniles without developing gametes corresponds to protein concentrations returning to low levels. The developing gametes of the "pseud juveniles" were found to be at a similar stage of development as gametes in the adults during May and June, yet no large increase in mean protein concentrations is seen in either males or females. This suggests that the presence of gametes in the juveniles is not solely responsible for the increase in protein concentration in June.

The appearance of juveniles with developing gametes and their subsequent disappearance in July was surprising, but can be explained in terms of migration. A number of studies have shown that *Arenicola marina* individuals migrate by burrowing or leaving their burrows and swimming (Newell, 1948; Beukema and de Vlas, 1979; Pollack, 1979). It is postulated that a cohort of juveniles had started to develop gametes in May and June and then migrated into the adult population by July. By July they had been replaced by another cohort of rapidly growing juveniles too small for collection in May and June, but large enough to be included in the samples taken in July.

In males, during June and July, mean protein concentrations increase. This is coincident with an increase in the percentage number of morula in these months as shown in Figure 4.7. However, the protein concentration decreases in August and then falls to the second lowest level in September even when there are still increases in the mean percentage number of morula in both of these months. Rashan (1980) also found an increase in protein concentration in pooled samples from males from May

through to October sampled from a population in Ireland. In May, mean protein concentration was  $0.16 \text{ mg.ml}^{-1}$  and this increased to  $2.9 \text{ mg.ml}^{-1}$  in October (Rashan, 1980). In neither August nor September did mean pooled protein concentrations fall as was the case in this study, although Rashan (1980) did find mean protein concentrations fell in October and November prior to spawning in December.

Such a drop in protein concentration specifically in September followed by a peak in November in this study cannot be easily explained. One possible explanation is the contamination of samples with blood in months when protein concentrations were high although this is very unlikely for all the months that have increased levels of protein concentration. From comparing Figure 4.5c and Figure 4.7 it would suggest that the increases in male protein concentration (excluding decreases in August and September) are related to increases in the percentage number of morula in males. However, although a significant weak positive correlation shown in Figure 4.7 between protein concentration and percentage number of morula is present, 75% of the variation in protein concentration is not accounted for by variation in the mean percentage number of morula. Rashan (1980), however, did correlate increases in the pooled mean protein concentration and numbers of protein fractions of coelomic fluid from males with increases in the percentage number of morula, although the variation between individuals was large.

In females, low levels of protein are maintained throughout the year except for an increase in October prior to spawning. The changes in protein concentration are only very weakly correlated with increases in mean oocyte diameter as shown in Figure 4.8. The increase in October could, however, be related to an accumulation of pre-CMF in the coelomic fluid prior to spawning. Data (not shown) shows that there is a modest correlation ( $R^2=0.583$ ) between protein concentration and mean oocyte diameter in October only, suggesting that the very mature females have higher levels of protein which may represent an accumulation in pre-CMF. In November the

majority of females have spawned and protein concentrations fall to levels similar to the rest of the year.

A correlation between increases in mean oocyte diameter and increases in protein concentration has been shown by Rashan (1980). Despite large variations between samples, protein concentrations and the number of protein fractions within the coelomic fluid were highest during the vitellogenesis stage of oocyte growth. Rashan (1980) suggested that these increases in protein concentration and the number of fractions in females may be due to the accumulation of proteins within the coelom from vitellogenic processes. It has already been discussed in section 1.2.1 that vitellogenesis in Nereids contains a heterosynthetic component. This involves the production of vitellogenin, the precursor of vitellin, from special coelomocytes in the coelomic fluid. Rashan (1980) postulated that a heterosynthetic component of vitellogenesis may exist in *Arenicola marina*. This would explain the increase in high molecular weight proteins within the coelom. However, results presented here fail to substantiate a correlation between protein concentration and gamete development in both males and females. The large variations between samples may be related only to the state of that individual worm and any trends in protein concentration within a population may be masked by individual variability. The presence of pre-CMF can also not be confirmed, but circumstantial evidence suggests that a precursor of CMF may be present in coelomic fluid prior to spawning.

The measurement of the protein concentration of individuals' coelomic fluid has not only been shown to vary greatly over a year but also within monthly samples, individual levels could vary greatly. The lack of a clear correlation between protein concentration and state of maturity may in some way be related to the choice of the Eden Estuary as the test population. The use of this population raised a number of difficulties in the examination of maturity in males, females and juveniles: Spawning in the Eden Estuary is not as precise as at other sites; occurring over a number of weeks in late autumn. This lack of synchronicity consequently meant that variation

between individuals was high especially in the months prior to or during the spawning (October and November). During these months, worms may have spawned and so have no gametes present or even have small developing gametes while other individuals may still be fully mature. This would therefore lower the mean percentage of morula and the mean oocyte diameter and increase standard error size. The distinction between adults and juveniles was also problematic so that in the adult population, individuals were found that were of similar size to the adults but had no or very small gametes present. This would again lower the overall mean of oocyte diameter and mean percentage number of morula and increase standard errors as these were classed as adults.

To achieve maximum maturation levels in oocytes of *Arenicola marina* a minimum exposure time to CMF of 20 minutes at 10°C has been shown to be necessary. This can be compared to a number of other different systems, but only in starfish can the oocyte maturation substance, 1-MeAde, be shown to have a similar role as CMF; that is another earlier substance is required to induce its production. In starfish the exposure time for 1-MeAde required to induce oocyte maturation has been termed the hormone dependent period (HDP) (Kanatani *et al.*, 1969). The HDP is temperature dependent; it increases as temperature decreases (Dorée and Guerrier, 1975). In *Marthasterias glacialis*, the maximum percentage maturation at 24°C is reached after 18 minutes (Guerrier and Dorée, 1975). This time is approximately the same for CMF, however, comparisons should be interpreted with caution. This is because of the probable differences in the mechanisms of maturation and the differences in incubation temperature. There are also wide variations in the HDP between batches of oocytes in starfish that also affect any comparisons between *A. marina* and other systems (Chiba and Hoshi, 1989).

The comparison of CMF with the action of 1-MeAde can also be examined in terms of the minimum concentrations required to induce maturation. In echinoderms, oocyte maturation is concentration independent above a threshold of  $10^{-7}$  M, although

this is dependent to some extent on the specific batch of oocytes used (Dorée *et al.*, 1976; Chiba and Hoshi, 1989). Although, the serial dilution of CMF would not give absolute values, the results presented in Figure 4.10 show that there is a strong dependence of oocyte maturation on dilution. The greater the dilution, the greater the reduction in numbers of oocytes maturing. However, the results fail to show any clear threshold below which any significant oocyte maturation occurs. Any threshold effect could be masked by the use of 6 oocyte donors each of which may have oocytes that have different response levels to CMF. Examination of the raw data (not shown) suggests that any threshold effects may be masked by this variation between females but also by variation between replicate counts.



## **Chapter 5**

### **The Properties and Chemical Nature of CMF in *Arenicola marina***

## 5.1 Introduction

The *in vivo* studies of Howie (1961a; 1963) contributed significantly to the understanding of the endocrine control in *Arenicola marina* males. It was not until an *in vitro* assay for sperm maturation was developed by Bentley (1985), however, that the final purification and isolation of the substance from the prostomium inducing spawning in males could be made. The Sperm Maturation Factor was purified and isolated and putatively identified as the fatty acid 8,11,14-eicosatrienoic acid (Pacey and Bentley, 1992a). This substance failed to induce spawning in females suggesting that the prostomial substance inducing spawning in females was not 8,11,14-eicosatrienoic acid (Bentley *et al.*, 1990; Pacey and Bentley, 1992a). Auckland's work in 1993, on the chemical properties of this unknown substance from female prostomia, was inconclusive. This was in part due to the lack of a reliable *in vitro* assay to assess the reactivity of fractions or treatments. No further progress has been made in the isolation and purification of the prostomial maturation substance.

The development of a reliable *in vitro* maturation assay for CMF as described in section 3.2 has not only confirmed its existence, but has enabled further aspects of CMF to be examined such as the data presented in Chapter 3 about its production and action. In this chapter the utilisation of the *in vitro* maturation assay for CMF has led to the examination of other characteristics of CMF, including its chemical nature and stability under different conditions. The experiments in this chapter fall into three broad categories; the activity of CMF under different conditions, levels of activity after specific purification steps and specific experiments to investigate the chemical class of CMF.

The investigation of the levels of activity of CMF before and after incubation under different conditions not only gives an idea of the stability of CMF, but also an indication of CMF's chemical structure.

The purification of a novel compound is important if the substance is to be characterised. In the case of CMF the presence of many other substances in the coelomic fluid makes purification an essential step. Purification steps by their nature can also give indications about the substance's chemical structure. Evaporation and subsequent rehydration of a substance can be used as a preliminary step in the purification of that substance by concentrating the substance's activity. This could then be detected by other techniques such as HPLC (High Performance Liquid Chromatography) and GC-MS (Gas Chromatography-Mass Spectrophotometer). The stability of a substance (whether the activity remains after the purification step) can also be examined and this can be used to make inferences about its structure. Sieving through molecular size filters is another useful purification technique that concentrates and gives an idea of a size of a molecule.

Other compounds were used in this study to gain evidence for a particular chemical class CMF may belong to. These were the use of tricyclic anti-depressants (TCAs) and the use of a non specific serine protease, trypsin. TCAs are psychotropic drugs; they block the uptake of amines such as serotonin, dopamine, noradrenaline, etc. by nerve terminals and affect neurotransmitter receptors such as serotonin receptors (Rang and Dale, 1991). TCAs were specifically used to see if incubation of oocytes in TCAs prior to incubation in CMF would inhibit oocyte maturation. In *Spisula solidissima* oocyte maturation is blocked by TCAs (Juneja *et al.*, 1993) and in *Dreissena polymorpha* TCAs inhibit spawning (Hardege *et al.*, in prep.). In both of these species oocyte maturation is induced by serotonin (Varaskin *et al.*, 1992; Fong *et al.*, 1994). The rationale behind the use of TCAs is that if CMF is serotonin, or a related amine, its action would be inhibited by incubating oocytes in TCAs first.

The same rationale exists for the use of proteolytic enzymes except that if CMF was a protein, consisting of arginine or lysine residues, then incubation of CMF with trypsin would breakdown the CMF molecule at these residues and inhibit its activity.

SDS-polyacrylamide gel electrophoresis (PAGE) can also estimate the molecular mass of proteins and peptides with more accuracy than just molecular mass sieves. Preliminary evidence from data in this chapter suggested that CMF may be proteinaceous. SDS-PAGE would enable an estimation of the molecular mass if CMF was indeed a protein.

## **5.2 The Activity of CMF under Different Conditions**

### **5.2.1 The Effects of Incubation for 1 Hour at Different Temperatures on the Activity of CMF**

#### **Materials and Methods**

For all experiments in this chapter *Arenicola marina* were collected, maintained, assessed for maturity, and had gametes removed as described in section 3.2.1. Prostomial homogenate preparation and injection, and coelomic fluid removal were also as described in section 3.2.1. The assessment of maturation using either unfixed or fixed oocytes and subsequent staining protocol was dependent on the time constraints for each experiment only.

#### **Experimental Design**

Six treatment groups were used, in each, 3  $\mu$ l of oocytes from each of 6 oocyte donors were each incubated in 50  $\mu$ l of coelomic fluid with CMF activity (harvested from 4 females and pooled as described in section 3.2.1). Coelomic fluid in Treatment 1 was assayed for CMF activity immediately after harvesting. The coelomic fluid of Treatments 2-6 were incubated at different temperatures shown in Table 5.1 for 1 hour. Each one was then assayed for CMF activity as described in section 3.2.1.

Oocytes were incubated directly in TFSW as a negative control to test for spontaneous maturation.

### Statistical Analysis

The nature of this data enabled statistical analysis to be performed using a two way analysis of variance on arcsine transformed data using the statistical package Minitab ver. 8.2. A multiple comparison Tukey test was used in breaking down these overall significant differences between treatments by comparing two treatments at a time.

Treatment	Incubation Temperature (1 hour)
2	0-0.5°C (on ice)
3	5°C (incubator)
4	10°C (cold room)
5	22°C (room temperature)
6	37°C (incubator)

Table 5.1. One hours incubation of CMF at different temperatures relevant to treatment number as referred to in the text.

### Results

The CMF activity of coelomic fluid (percentage number of oocytes maturing) was compared after incubation at different temperatures for 1 hour and these were also compared to coelomic fluid which was assayed immediately. The results are shown including the sea water control in Figure 5.1.

Analysis shows that there are very significant differences between all treatments, including the sea water control, in the number of oocytes matured in each treatment ( $F=841.28$ ,  $p<0.001$ ) and very significant differences between the ability of oocytes of the six females used to undergo induced maturation ( $F=350.13$ ,  $p<0.001$ ).

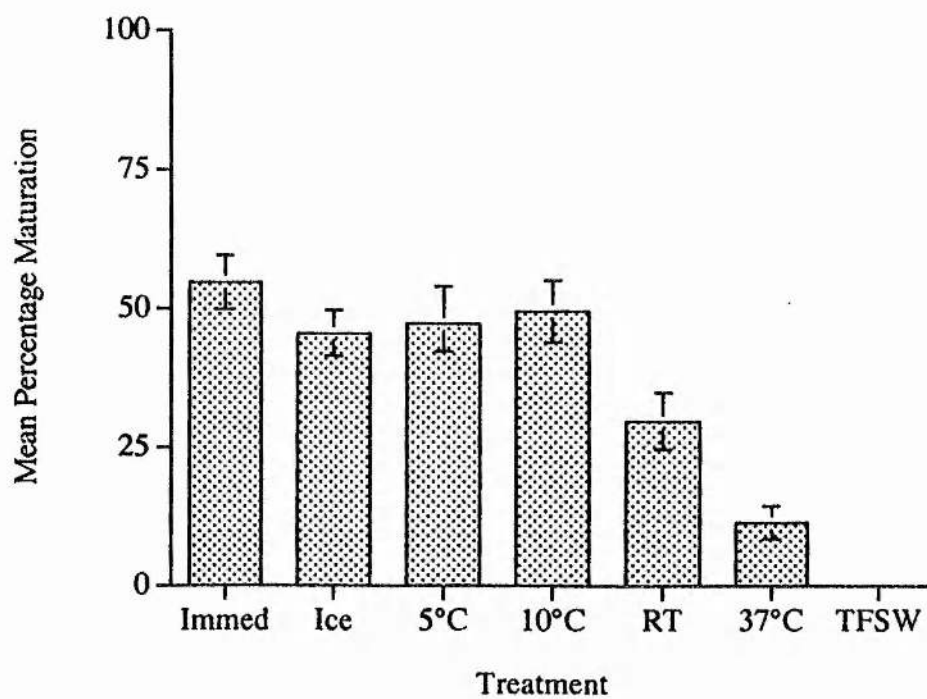


Fig. 5.1. Response of *Arenicola marina* oocytes of 6 oocyte donors to incubation in different treatments: Immed, immediate incubation in coelomic fluid containing CMF; Ice, incubation in coelomic fluid containing CMF after 1 hours incubation of the coelomic fluid on ice (0.5 °C); 5°C, 10°C, RT (room temperature [22°C]), 37°C, incubation in coelomic fluid containing CMF after 1 hours incubation of the coelomic fluids at specified temperatures; TFSW, TFSW only. All oocytes were incubated for 3 hours at 10°C. Response expressed as arcsine back-transformed mean percentage ( $\pm$ S.E.M.) of oocytes matured. Three replicates of 50 oocytes counted from each oocyte donor.



Significant interaction is also present between females and treatments ( $F=27.55$ ,  $p<0.001$ ). Although there are significant differences between females and significant interaction effects, only differences between treatments are important. To examine differences between treatments only, a multiple comparison (Tukey Test) was performed and the results are summarised in Table 5.2. Significant differences ( $p<0.001$ ) are present between all pairs of treatments including the sea water control except between all ways comparisons of Treatments 2,3 and 4 (coelomic fluid with CMF incubated on ice, at 5°C and 10°C respectively). There is also no significant difference, between Treatment 1 with Treatment 4 (coelomic fluid with CMF assayed immediately and coelomic fluid with CMF incubated at 10°C).

### **5.2.2 The Effect of Heating on the Activity of CMF**

#### **Experimental Design**

Coelomic fluid with CMF activity was harvested from a single female injected with homogeante containing 1 prostomium equivalent as described in section 3.2.1. A 1 ml sample of coelomic fluid with CMF activity was immediately frozen at -20°C. The sample was frozen for 1 day before being defrosted and divided in two. Three treatment groups were used, in each, oocytes from 6 oocyte donors were incubated in the fractions to be tested. Each fraction was then assayed for CMF activity as described in section 3.2.1.

#### *Treatment 1*

Five hundred microlitres (50%) of coelomic fluid with CMF activity centrifuged for 30 seconds at 6500 rpm.

Treatment							
1 (Immed)	*****						
2 (Ice)	<0.001	*****					
3 (5°C)	<0.001		*****				
4 (10°C)				*****			
5 (22°C)	<0.001	<0.001	<0.001	<0.001	*****		
6 (37°C)	<0.001	<0.001	<0.001	<0.001	<0.001	*****	
TFSW (Control)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	*****
	1 (Immed)	2 (Ice)	3 (5°C)	4 (10°C)	5 (22°C)	6 (37°C)	TFSW (Contro)

Table 5.2. Matrix showing significant comparisons between two treatments and level of significance from multiple comparison Tukey Test on the effect of 1 hours incubation at different temperatures on the activity of CMF.

*Treatment 2*

Five hundred microlitres (50%) of coelomic fluid with CMF activity was heated in distilled water at 95°C for 1 minute and then centrifuged for 30 seconds at 6500 rpm. The supernatant was removed and this was assayed for maturational activity as described above.

*Treatment 3*

Oocytes were incubated with 500  $\mu$ l of TFSW as a negative control.

**Statistical Analysis**

Statistical analysis was carried out using generalised linear interactive modelling (GLIM Version 3.77). Interaction effects were also included in the analysis, but further analysis of the data with Student T-tests to examine differences between each treatment could not be completed because of overparametisation of the data within the model. Comparisons of differences between individual treatments were therefore carried out by graphical inspection only.

**Results**

The results from this experiment are shown in Figure 5.2. The effect of heating on active coelomic fluid for 1 minute at 95°C is to induce a significant and complete loss of CMF activity. Very significant differences are present between all treatments ( $F=7283$ ,  $p<0.001$ ) and between the ability of oocytes of the six females used to undergo induced maturation ( $F=53.56$ ,  $p<0.001$ ). There are no significant interaction effects between females and treatments ( $F=0.00$ ,  $p>0.05$ ). Graphical analysis shows that the activity present in untreated coelomic fluid with CMF activity (Treatment 1) is significantly greater than either coelomic fluid with CMF activity which had been heated (Treatment 2) or the negative control (Treatment 3) which are not significantly different from each other.

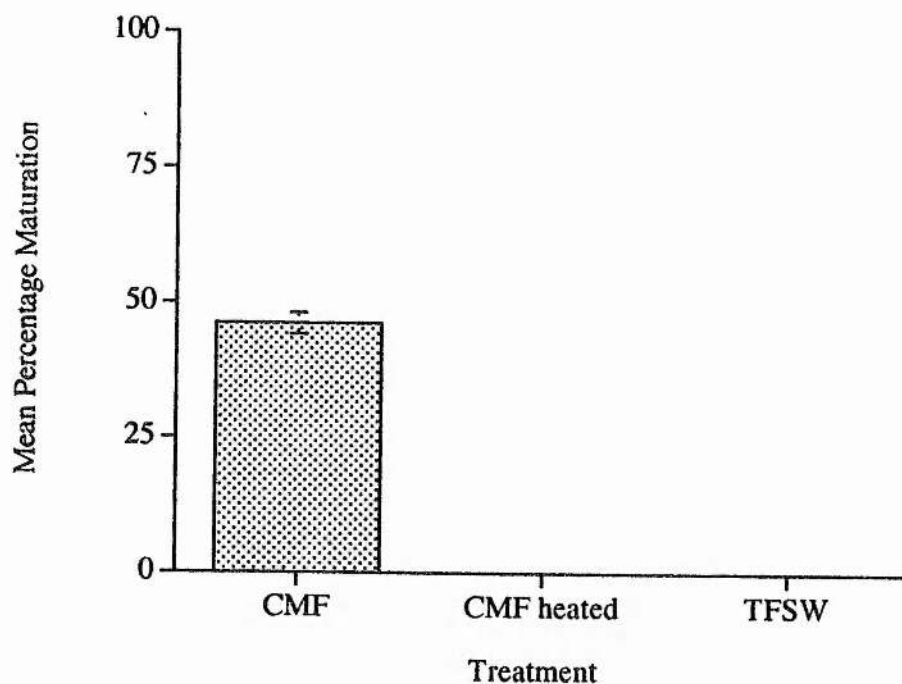


Fig. 5.2. Response of *Arenicola marina* oocytes of 6 oocyte donors to incubation in different treatments: CMF, coelomic fluid containing CMF; CMF Heated, coelomic fluid containing CMF after heating for 1 minute at 95°C; TFSW, TFSW only. All incubations were carried out at 10°C for 3 hours. Response expressed as arcsine back-transformed mean percentage ( $\pm$ S.E.M.) of oocytes matured. Three replicates of 50 oocytes counted from each oocyte donor.

## 5.3 Purification of CMF

### 5.3.1 The Effect of Evaporation and Subsequent Rehydration of Coelomic Fluid on CMF Activity

#### Experimental Design

Four treatment groups were used, in each, oocytes from 6 oocyte donors were incubated in the treatments to be tested. Coelomic fluid with CMF activity was harvested from one female and then all treatments were assayed for CMF activity as described in section 3.2.1

#### *Treatment 1*

Fifty percent (350  $\mu$ l) of the coelomic fluid with CMF activity was incubated with oocytes (positive control).

#### *Treatment 2*

Fifty percent (350  $\mu$ l) of the coelomic fluid with CMF activity was placed in an Eppendorf tube with holes pierced in the lid with a 21 g syringe needle, which was then placed in a Genevac SF-50 centrifugal concentrator. The sample was concentrated under vacuum while spinning the sample at approximately 200 g. The sample was spun for 2.5 hours before being rehydrated with an equivalent volume of distilled water and then vortexed for 30 seconds to ensure complete rehydration and mixing.

#### *Treatment 3*

For a negative control, 350  $\mu$ l of TFSW was also concentrated and rehydrated as described for Treatment 2.

#### *Treatment 4*

Oocytes were incubated directly in TFSW as a negative control to test for spontaneous maturation.

### **Statistical Analysis**

Statistical analysis was carried out using generalised linear interactive modelling (GLIM Version 3.77). Interaction effects were included in the analysis, but further analysis of the data with Student T-tests to examine differences between each treatment could not be completed because of overparametisation of the data within the model. Comparisons of differences between individual treatments were therefore carried out by graphical inspection only.

### **Results**

The results of this experiment are shown in Figure 5.3. Analysis shows that there are highly significant differences in the levels of CMF activity between all treatments including the sea water control ( $F=2660$ ,  $p<0.001$ ) and highly significant differences between the ability of oocytes of the six females used to undergo induced maturation ( $F=209.6$ ,  $p<0.001$ ). Significant interaction is also present between females and treatments ( $F=20.3$ ,  $p<0.001$ ). Graphical examination of the data clearly shows that after evaporation and rehydration of active coelomic fluid there is a very significant reduction in CMF activity. After this purification step, a small but significant amount of activity remains when compared to both controls.

#### **5.3.2 Assessment of CMF Activity after Filtration Through a 10 kilodalton Filter**

##### **Experimental Design**

Four treatment groups were used, in each, oocytes from 6 oocyte donors were incubated in the each of the treatments to be tested. Coelomic fluid with CMF activity



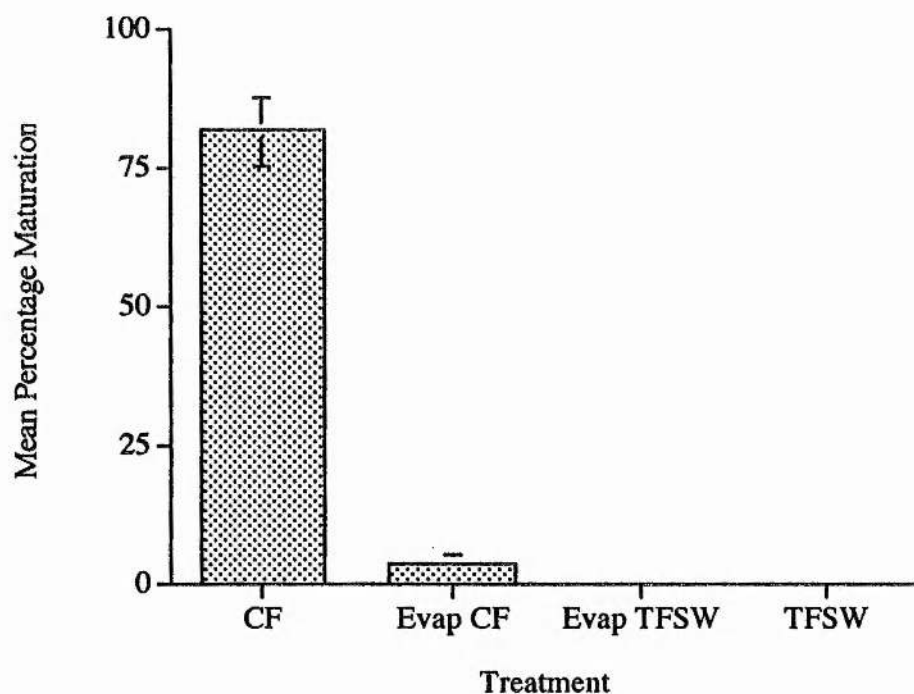


Fig. 5.3. Response of *Arenicola marina* oocytes of 6 oocyte donors to incubation in different treatments: CF, coelomic fluid containing CMF; Evap CF, evaporation and subsequent rehydration of coelomic fluid containing CMF; Evap TFSW, evaporation and subsequent rehydration of TFSW; TFSW, TFSW only. All incubations were carried out at 10°C for 3 hours. Response expressed as arcsine back-transformed mean percentage ( $\pm$ S.E.M.) of oocytes matured. Three replicates of 50 oocytes counted from each oocyte donor.

was harvested and pooled from 4 females and all treatments were assessed for CMF activity as described in section 3.2.1.

#### *Treatment 1*

Fifty percent (2.5 ml) of the coelomic fluid with CMF activity was incubated with donor oocytes.

#### *Treatment 2*

Fifty percent (2.5 ml) of the coelomic fluid with CMF activity was filtered through a 10 kDa membrane filter. The filter system used was a Stirred Ultrafiltration Cell 8050 on a magnetic stirrer with 10 kDa Diaflo® membrane Ultrafilter (Amicon Inc.) using dry nitrogen at pressure of 3.7 atmospheres.

#### *Treatment 3*

The membrane filter used to filter the coelomic fluid in Treatment 2 was washed with 2.5 ml of TFSW for 2 minutes. This wash was then assayed for CMF activity as described in section 3.2.1.

#### *Treatment 4*

Oocytes were incubated with 2.5 ml of TFSW as a negative control.

### **Statistical Analysis**

Statistical analysis was carried out using generalised linear interactive modelling (GLIM Version 3.77). Inclusion of interaction effects resulted in overparametisation of the data within the model. Consequently, interaction effects were ignored so that Student T-tests could then be performed on the data to examine differences between each treatment.

## Results

The results in Figure 5.4 show that CMF activity is present in active coelomic fluid (Treatment 1) but this is lost after passing through a 10 kDa filter (Treatment 2). Activity is partially recovered after the filter was washed (Treatment 3) Analysis shows that there are significant differences in levels of CMF activity between treatments ( $F=144.3$ ,  $p<0.001$ ) and between the ability of oocytes of the six females used to undergo induced maturation ( $F=12.18$ ,  $p<0.001$ ). Student T-tests used to show up differences between pairs of treatments show that the activity present in the active coelomic fluid is significantly higher than the sea water control ( $t=23.686$ ,  $p<0.001$ ). The activity within the active coelomic fluid is significantly reduced ( $t=24.718$ ,  $p<0.001$ ) after filtration, to a level that is not significantly different from the sea water control ( $t=0.815$ ,  $p>0.05$ ). The CMF activity, partially regained after washing the filter, is significantly lower ( $t=19.44$ ,  $p<0.001$ ) than the original amount of CMF activity from the coelomic fluid, but is also significantly greater than the sea water control ( $t=13.866$ ,  $p<0.001$ ).

## 5.4 The Chemical Nature of CMF

### 5.4.1 Assessment of the Effects of Tricyclic Anti-depressants (TCAs) on the Maturational Ability of Oocytes

#### Experimental Design

All incubations were performed in Eppendorf tubes. Twenty  $\mu\text{l}$  of oocytes (removed from a number of different oocyte donors and pooled as described in section 3.2.1) were added to 1 ml of each chemical concentration and maintained in the dark (due to the photo-sensitive nature of the chemical, imipramine) at  $10^{\circ}\text{C}$  for 1 hour. Artificial sea water (ASW) was used for all solutions (see appendix). All chemicals

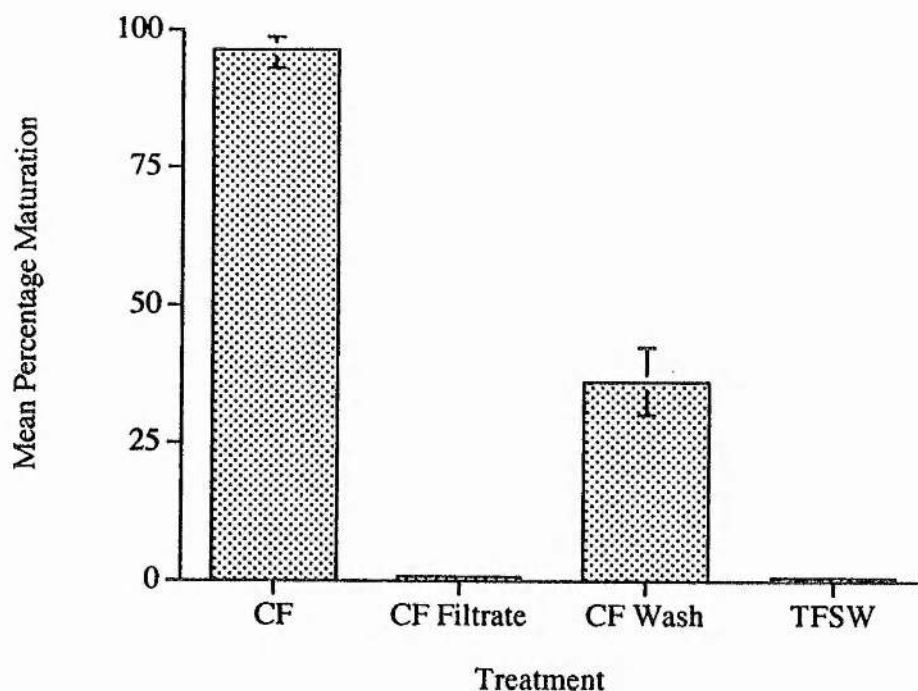


Fig. 5.4. Response of *Arenicola marina* oocytes of 6 oocyte donors to incubation in different treatments: TFSW, TFSW only; CF, coelomic fluid containing CMF; CF Filtrate, coelomic fluid with CMF after filtration through 10 kDa filter; CF Wash, 10 kDa filter washed with TFSW. All purification steps were carried out at 22°C. All incubations of oocytes were for 3 hours at 10°C. Response expressed as arcsine back-transformed mean percentage ( $\pm$ S.E.M.) of oocytes matured. Three replicates of 50 oocytes counted from each oocyte donor.

were dissolved in ethanol and then subsequently diluted in ASW to give a final maximum ethanol concentration of 0.1%.

After incubation in the chemical solutions, the oocytes were removed and washed three times in TFSW. The oocytes were subsequently incubated in 50  $\mu$ l of coelomic fluid with CMF activity in a 96 well plate for 3 hours at 10°C under ambient illumination. Oocytes were then removed and fixed and the maturational state of oocytes was then assessed using the fluorescence assay as described in section 2.2.1. All chemicals were tested at 3 concentrations;  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  M. All chemicals were purchased from Sigma Co. The oocytes were incubated in a number of treatments:

#### *Positive Control*

Coelomic fluids of six females (1-6) with CMF activity, harvested as described in section 3.2.1 (to establish that oocytes had the ability to mature).

#### *Negative Controls*

- a) ASW (to detect any spontaneous maturation of the oocytes)
- b) 0.1% ethanol in ASW (to control for any possible effects of ethanol)

#### *Chemicals Tested for Inhibition of Ability of Oocytes to Mature in the Presence of CMF Activity*

- a) Clomipramine
- b) Desipramine
- c) Imipramine

#### **Statistical Analysis**

Statistical analysis was carried out using a Kruskal Wallis Test on all treatments excluding the sea water control.

## Results

The results presented in Figure 5.5 show the effect of incubating oocytes in different TCAs on the subsequent ability of those oocytes to mature when exposed to CMF activity. From graphical analysis only, all treatments induced significant maturation in oocytes compared to the sea water control. Analysis using the Kruskal Wallis Test shows that there are no significant differences ( $H=13.063$ ,  $p>0.05$ ) between the ability of oocytes to mature between all treatments, excluding the sea water control. Very significant differences between females are, however, present ( $H=146.880$ ,  $p<0.001$ ).

### 5.4.2 Assessment of CMF Activity after Incubation with Trypsin

#### Experimental Design

Pooled active coelomic fluid (harvested from 4 females as described in section 3.2.1) was divided between four treatments as shown below. Oocytes from 5 oocyte donors were used to assess CMF activity of the coelomic fluid after each treatment. Oocytes were also incubated directly in TFSW as a control. Assessment of CMF activity was as described in section 3.2.1. The coelomic fluid fractions were incubated with the treatments at 22°C (room temperature) under ambient illumination. To ensure complete mixing each coelomic fluid was vortexed for 10 seconds after each solution was added. All chemicals were purchased from Sigma Co.

#### *Treatment 1*

Twenty five percent of the active coelomic fluid (250  $\mu$ l) was incubated for 30 minutes under ambient illumination and 22°C (positive control).



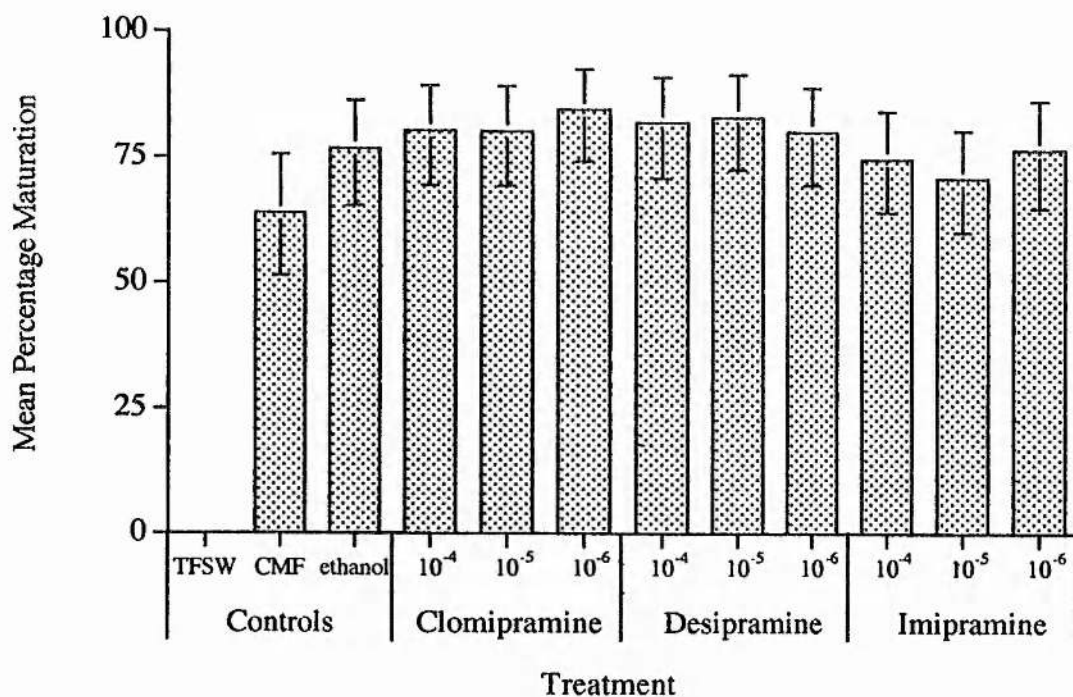


Fig. 5.5. Response of *Arenicola marina* oocytes of 5 oocyte donors to incubation in coelomic fluid with CMF activity after pre-treating the oocytes for 1 hour in different treatments: CMF, TFSW only; Ethanol, 1% Ethanol in TFSW; Clomipramine; Desipramine; Imipramine were all tested at  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  M concentrations. All pre-treatments were performed in the dark at  $10^{\circ}\text{C}$ . All incubations in coelomic fluid with CMF and in the control of TFSW only, were for 3 hours at  $10^{\circ}\text{C}$ . Response expressed as arcsine back-transformed mean percentage ( $\pm$ S.E.M.) of oocytes matured. Three replicates of 50 oocytes counted from each oocyte donor.

*Treatment 2*

Twenty five percent of the active coelomic fluid (250  $\mu$ l) was incubated with 10  $\mu$ l of trypsin solution (see appendix) for 15 minutes at 22°C. To this was added 10  $\mu$ l of trypsin inhibitor solution (see appendix) and incubated for a further 15 minutes at 22°C.

*Treatment 3*

Twenty five percent of the active coelomic fluid (250  $\mu$ l) with CMF activity was incubated with 10  $\mu$ l of trypsin inhibitor solution for 30 minutes at 22°C under ambient illumination.

*Treatment 4*

Twenty five percent of the active coelomic fluid (250  $\mu$ l) with CMF activity was incubated with 10  $\mu$ l of trypsin/trypsin inhibitor solution for 30 minutes at 22°C under ambient illumination. This was made up 5 minutes before use with equal volumes of trypsin solution and trypsin inhibitor solution (see appendix).

**Statistical Analysis**

Statistical analysis was carried out using generalised linear interactive modelling (GLIM Version 3.77). Interaction effects were excluded from the analysis and further analysis of the data with Student T-tests was not performed because the nature of the data resulted in overparametisation within the model. Comparisons of differences between individual treatments were therefore carried out by graphical inspection only.

**Results**

The results from this experiment are shown in Figure 5.6. Highly significant differences are present between levels of CMF activity for all treatments ( $F=256$ ,

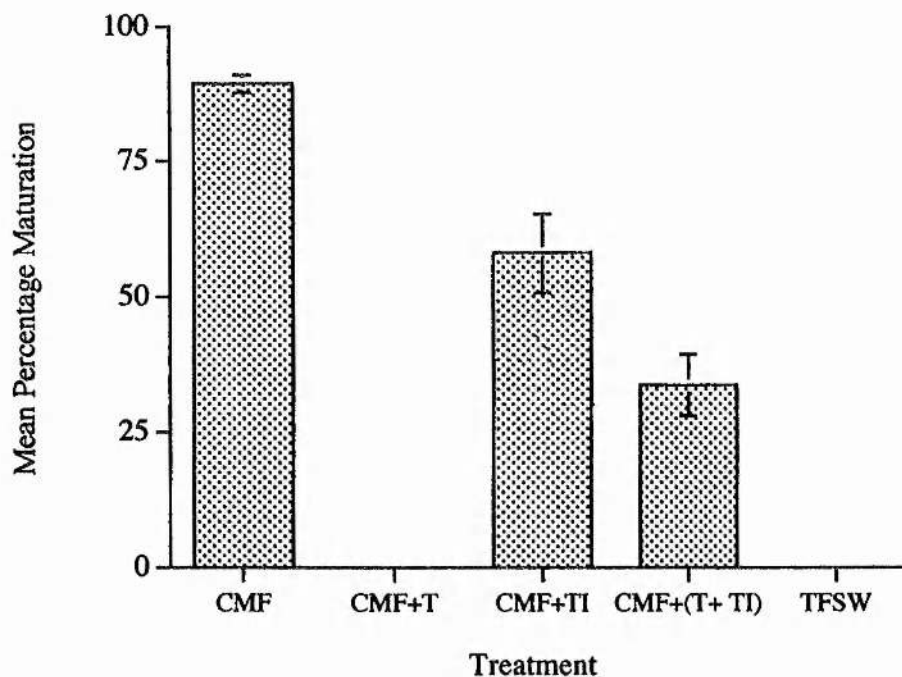


Fig. 5.6. Response of *Arenicola marina* oocytes of 5 oocyte donors to incubation in different treatments: CMF, coelomic fluid with CMF for 30 minutes; CMF+T, coelomic fluid with CMF after incubation in trypsin for 15 minutes and then the addition of trypsin inhibitor for 15 minutes; CMF+TI, coelomic fluid with CMF after incubation in trypsin inhibitor for 30 minutes; CMF+(T+TI), coelomic fluid with CMF after incubation in premixed trypsin and trypsin inhibitor for 30 minutes; TFSW, TFSW only. All incubations of coelomic fluid with treatments were carried out at 22°C. All incubations of oocytes in coelomic fluid were for 3 hours at 10°C. Response expressed as arcsine back-transformed mean percentage ( $\pm$ S.E.M.) of oocytes matured. Three replicates of 50 oocytes counted from each oocyte donor.

$p < 0.001$ ) and between the ability of oocytes of the six females used, to undergo induced maturation ( $F=25.3$ ,  $p < 0.001$ ). Graphical analysis shows there is significant activity present in Treatment 1 in comparison to the sea water control. This activity is completely lost after incubation with trypsin solution (Treatment 2). Activity is also significantly reduced when active coelomic fluid is incubated with trypsin inhibitor only (Treatment 3). Nevertheless, significant activity remains after incubation with trypsin inhibitor only (Treatment 3), when compared to the incubation with trypsin only solution (Treatment 2). Activity is also significantly reduced when active coelomic fluid was incubated with pre-mixed trypsin and trypsin inhibitor (Treatment 4). Again, however, significant activity remains in the coelomic fluid incubated with pre-mixed trypsin and trypsin inhibitor (Treatment 4) when compared to incubation with trypsin only solution (Treatment 2). This level of activity is significantly less than activity that remained after incubation with trypsin inhibitor only (Treatment 3).

#### **5.4.3 Sodium-dodecyl-sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of CMF**

##### **Materials and Methods**

##### **Experimental Design**

Prior to the injection of 200  $\mu\text{l}$  of homogenate containing 1 prostomium equivalent into 20 females, and the injection of 200  $\mu\text{l}$  of TFSW into 10 control females, each female had 200  $\mu\text{l}$  of coelomic fluid removed as described in section 3.2.1. Once injection was complete, 200  $\mu\text{l}$  of coelomic fluid was removed from each female at c. 2 hours post-injection. Sub-samples of 50  $\mu\text{l}$  of all these coelomic fluid samples, both pre and post-injection, were assessed for CMF activity with pooled oocytes from a number of oocyte donors as described in section 3.2.1. The remaining 150  $\mu\text{l}$  of each sample of coelomic fluid, pre and post-injection, and 150  $\mu\text{l}$  of the prostomial homogenate (used to induce the production of CMF in the experiment)

were added to 50  $\mu$ l of SDS buffer (see appendix), which had been warmed to 80°C for 4 minutes. All samples were then stored at -20°C until required for use.

SDS-PAGE (Sodium-dodecyl-sulphate Polyacrylamide-Gel Electrophoresis) of samples was carried out as described by Laemmli (1970). Fifteen percent acrylamide gels were loaded with 2  $\mu$ g of protein in 10  $\mu$ l of sample and then stained with Coomassie Brilliant Blue G-250 (Neuhoff *et al.*, 1988) and silver (Bloom *et al.*, 1987).

## Results

Four females with the most CMF activity in the post-injection coelomic fluid (data not shown) and their corresponding pre-injection coelomic fluid samples were chosen for the gel runs. These eight samples as well as the prostomial homogenate sample and a Dalton VII marker (Sigma Co.) were then run on three gels of 10, 15 and 20% acrylamide to visualise all proteins present. A photograph of the 15% acrylamide one-dimensional SDS PAGE gel of these samples is shown in Figure 5.7. Lane 1 is the dalton marker. Seven markers are present, of molecular masses ranging from 14.2 to 66 kDa (as indicated on Figure 5.7). Lanes 2 and 3 are runs of coelomic fluid from female 1, pre and post-injection of homogenised prostomia respectively. Lanes 4 and 5 are runs of coelomic fluid from female 2, pre and post-injection of homogenised prostomia respectively, as are lanes 6 and 7 and 8 and 9, but from females 3 and 4 respectively. Column 10 is of pure prostomial homogenate injected into the females.

The results indicate that in females 1 and 2, a number of bands around the 36 and 45 kDa markers are present in the pre-injection coelomic fluid which disappear after injection. However this pattern is not repeated in females 3 and 4 where these bands are still present in the post-injection coelomic fluid. Bands present in lane 10 (prostomial homogenate) are also present in pre and post-injected coelomic fluids from all females. The 10% and 20% gels (not shown) show that no other bands were



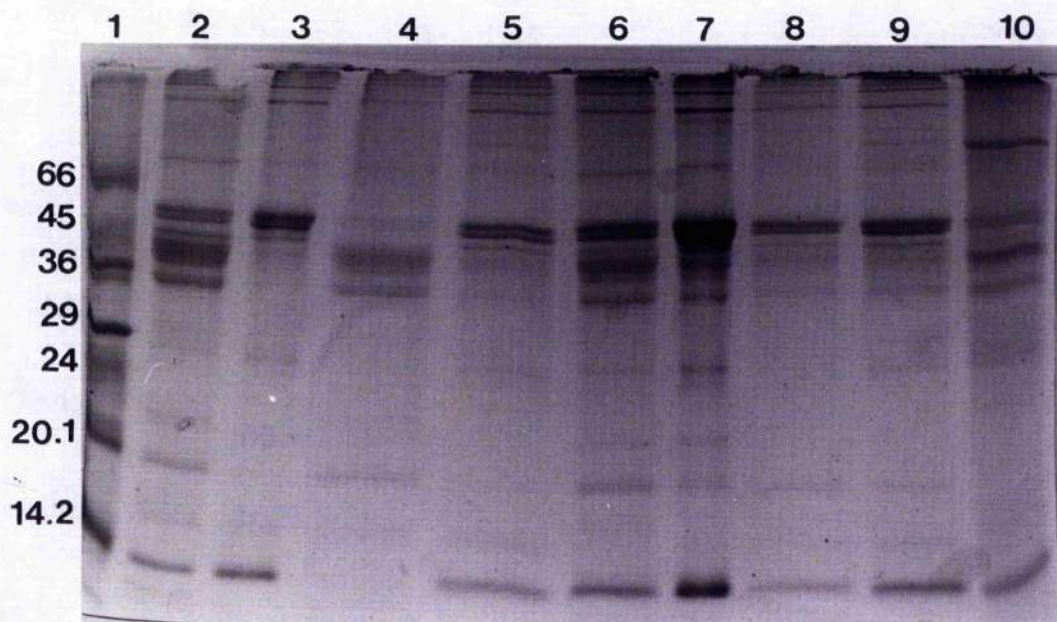


Fig. 5.7. One-dimensional SDS-PAGE gel with 15% acrylamide. Samples were loaded onto the gel at a protein concentration of  $2 \mu\text{g}.\text{ml}^{-1}$  and stained with Coomassie blue. Lane 1, Dalton VII marker with markers at 66, 45, 36, 29, 24, 20.1 and 14.2 kilodaltons; Lane 2, coelomic fluid removed from female 1 just prior to the injection of prostomial homogenate; Lane 3, coelomic fluid from female 1 removed 2 hours after the injection of homogenised prostomia; Lanes 4 and 5, 6 and 7 and 8 and 9 are the same as lanes 2 and 3 except coelomic fluid was removed from females 2, 3, and 4 respectively; Lane 10, pure prostomial homogenate of a concentration of 1 prostomium equivalent.



visualised below or above the range of bands found in the 15% gel. The results fail to show any clear pattern for pre and post-injected coelomic fluid in relation to the presence of a band relating to CMF.

## 5.5. Discussion

### 5.5.1 The Stability of CMF

The results described in section 5.2 show clearly that CMF activity is susceptible to increases in temperature. A significant reduction in CMF activity resulted after one hour's incubation at any of the tested temperatures except 10°C. No significant differences in the levels of CMF activity are present after incubation at 0.5°C (ice), 5°C or 10°C. Incubation of CMF at room temperature (22°C) for 1 hour, however, results in a significant loss of activity when compared to incubation at 0.5°C (ice), 5°C or 10°C, with another significant decrease when incubated at 37°C. Optimal CMF activity remains after incubation at temperatures of 10°C or below. CMF activity is therefore most stable at a physiological temperature for *Arenicola marina*. Any increase in temperature results in loss of CMF activity and this is reflected in the effects of higher temperatures on the metabolic processes of the whole worm: in populations that spawn in autumn and early winter, maintaining worms at greater than 10°C in the laboratory results in high mortality rates in comparison to worms maintained at 10°C or below (pers. obser.). CMF activity is also completely lost after incubation at temperatures of 95°C, confirming the thermolabile nature of CMF.

Both these results not only confirm the unstable nature of CMF and that it is thermolabile, but can also give some clues to its chemical structure. Most large proteins are denatured after heating to 95°C. The thermolabile nature of CMF suggests that it may have a protein structure although many other active molecules would be inactivated by heating to this temperature.

### 5.5.2 Purification of CMF

The purification steps performed in section 5.3 are not only purification steps but also provide interesting information about the nature of CMF. Firstly, the unstable nature of CMF is further reinforced by the loss of activity after evaporation and subsequent rehydration. The loss of activity during this technique also means that as a purification step this may not be the best method, unless conditions during the process can be manipulated to prevent loss of activity.

The use of a 10 kDa filter as a purification technique is feasible because activity is retained on the filter and can be removed by washing. More importantly, however, the loss of CMF activity after passing active coelomic fluid through the 10 kDa filter, which is partially regained after washing the filter, indicates that CMF has a molecular mass of greater than 10 kDa.

### 5.5.3 Chemical Nature of CMF

The complete inactivation of CMF by trypsin, which specifically cleaves arginine or lysine residues, provides the most substantial evidence that CMF is a protein. Although CMF activity is partially inhibited by the addition of trypsin inhibitor only, there is not a complete loss of activity as is the case with just trypsin. This is also true for pre-mixed trypsin and trypsin inhibitor when added to CMF. The reduction in activity is significantly greater in this case than just trypsin inhibitor but again significant activity still remains. The loss of activity when trypsin inhibitor is added is surprising and why it inhibits CMF cannot be easily explained. The loss of activity due to the addition of pre-mixed trypsin and trypsin inhibitor may be due to the fact that the trypsin and trypsin inhibitor had not been mixed for enough time prior to the addition to CMF so that some trypsin was still free to inhibit the CMF.

CMF's inactivation by trypsin, its thermolabile nature and a molecular size of greater than 10 kDa suggests a proteinaceous chemical structure. SDS-gel

electrophoresis separates out protein subunits by electrophoretic mobility (which is a function of size and charge) and was used firstly to give an indication of the molecular mass of CMF. Secondly, it was used to investigate if CMF was produced *de novo* in coelomic fluid or was produced from other proteins. By comparing coelomic fluid with no CMF activity (pre-injected) and coelomic fluid with CMF activity (post-injected with prostomial homogenate) and pure prostomial homogenate, the gels would show up any new or modified bands that could be ascribed to CMF production. However, the gels failed to produce consistent results between females and so no conclusions can be drawn about CMF. It is likely that the presence of blood and other proteins within the coelomic fluid masked CMF, for example in lane 7 (post-injection coelomic fluid of female 3) the heavy banding is likely to be due to blood. The relative concentrations of CMF may also have been too small to resolve on the gel.

TCAs are known to inhibit oocyte maturation in the mollusc, *Spisula solidissima* (Juneja *et al.*, 1993) but at similar concentrations TCAs fail to inhibit maturation in *Arenicola marina*. The action of TCAs is known to block the uptake of amines such as serotonin, dopamine, noradrenaline etc. by nerve terminals and also affect neurotransmitter receptors such as serotonin receptors (Rang and Dale, 1991). The exact mechanism of oocyte maturation inhibition in *S. solidissima* is unknown, but TCAs may modulate the action of serotonin by blocking calcium channels in the oocyte (Juneja *et al.*, 1994). The lack of inhibition of oocyte maturation by TCAs and data from Chapter 7 that serotonin fails to induce oocyte maturation suggests that in *Arenicola marina* the mechanism of maturation is not based on serotonin or other amines.

The results obtained in this study make it possible to begin to get an overall picture of the properties and chemical structure of CMF. These appear to be very different from the majority of maturation inducers already documented. The chemical structure of substances involved in oocyte maturation in a variety of phyla are now

known to be diverse (see Introduction). For example, the maturation inducer in starfish is 1-methyl adenine (Kanatani *et al.*, in 1969), while the amine, serotonin was confirmed as the maturation inducer in the mollusc *Spisula* (Hirai *et al.*, 1984). In the hydrozoan, *Spirocodon saltarix*, a peptide is thought to be involved (Freeman, 1987), while in amphibians it is the steroid, progesterone (Baulieu and Schorderet-Slatkine, 1983). The experiments have shown CMF to have qualities that are different from most maturation inducers already documented, that is proteinaceous in nature. The evidence for this, although not conclusive, is strong when taken together; a molecular mass greater than 10 kDa, thermolabile and unstable during evaporation. Most importantly CMF is inactivated by trypsin which specifically requires an arginine or lysine residue to cleave.

All these experiments indicate that CMF has different characteristics to those of other maturation inducing substances that have been described in other groups. However, CMF does show similarities with a maturation inducing substance found in the sub-oesophageal ganglion and cement gland complex of the polychaete *Pectinaria gouldii* (Tweedell, 1980). This substance was also found to be thermolabile and have a molecular mass of greater than 12 kDa, although it was not found to be sex specific (Tweedell, 1980).

## **Chapter 6**

### **The Mode of Action of CMF in Oocyte Maturation in *Arenicola marina***

## 6.1 Introduction

The investigation of the mode of action of CMF upon oocytes of *Arenicola marina* also requires two questions to be asked; i) What chemical pathways are involved during the process of maturation and ii) on which of these pathways does CMF exert its effects on or within the oocyte. One method used to gain an insight into the pathways involved in maturation is the use of chemicals that have known actions, and to examine their ability to induce oocyte maturation *in vitro*. Work by Meijer (1980), discussed in section 1.3.2, investigated the maturation response of oocytes of *Arenicola defodiens*, to certain chemicals. Preliminary work by Auckland (1993), using chemicals to induce oocyte maturation in *A. marina*, suggested that some differences in maturation may exist between the two species and this is investigated further in this chapter.

The chemicals used in this study have all been shown to be capable of inducing maturation in oocytes of at least one species. The mechanisms by which each of these chemicals exerts their action are diverse, but can be grouped according to their known targets.

A number of chemicals used in this study have an effect on calcium levels and calcium transport in the cell. The ionophore A23187 facilitates the transportation of divalent cations across membranes so increasing the internal calcium concentration (Reed and Lardy, 1972). It was found to induce maturation in *Spisula* sp. (Schuetz, 1975) and *Pectinaria gouldii* (Anstrom and Summers, 1981), but failed to induce maturation in *Arenicola defodiens* (Meijer, 1980) and starfish (Nemoto, 1982). However, it did have an additive effect with 1-MeAde by reducing the hormone dependent period (Nemoto, 1982). The local anaesthetics, tetracaine and its weaker analogue procaine, displace calcium from membranes and release calcium from membrane phospholipid complexes such as phosphatidyl-L-serine and phosphatidylethanolamine (Seeman, 1972). Both compounds induce maturation in



*A. defodiens* (Meijer, 1980) and in *Chaetopterus* sp. (Ikegami *et al.*, 1976) although procaine is less active than tetracaine in *A. defodiens*. The  $\beta$ -adrenoceptor antagonists, oxeprenolol and propranolol, increase intracellular calcium levels by displacing calcium ions adsorbed to phospholipid complexes in the membrane. This results in the hyperpolarisation of the membrane and further uptake of calcium (Szasz, 1977). Both of these chemicals induce maturation in *A. defodiens*. The  $\text{La}^{3+}$  ion of lanthanum chloride also induces maturation in *A. defodiens*. This is a specific antagonist of calcium in biological systems and displaces calcium from specific sites increasing the free levels of  $\text{Ca}^{2+}$  ions (Weiss, 1974).

The compounds DL-dithiothreitol (DTT) and 2,3-dimercaptoethanolis are a disulphide reducing agents. These compounds were reported to induce maturation in *Arenicola defodiens* and in other groups such as starfish (Kishimoto and Kanatani, 1973; Meijer, 1980). They are thought to act on the surface of the oocyte as injection of DTT fails to induce maturation in starfish (Kishimoto and Kanatani, 1973).

The action of serotonin on inducing maturation in some molluscs has already been discussed in detail in section 1.3.5. The incubation of *Arenicola marina* oocytes in serotonin was to determine whether the mechanisms of maturation involving serotonin in these other species were similar for *Arenicola marina*.

## 6.2 Materials and Methods

For this chapter, *Arenicola marina* were collected, maintained and assessed for maturity as described in section 3.2.1.

### 6.2.1 Experimental Design

As a positive control, coelomic fluid with CMF activity was obtained by the injection of 6 females with homogeante containing 1 prostomium equivalent each as described in section 3.2.1. Two hours after injection, coelomic fluid was removed from individual worms as described in section 3.2.1 and was stored on ice until required. Incubation of oocytes in the positive controls was for 4 hours at 10°C in the dark. Three microlitres of oocytes were incubated in 50  $\mu$ l of each of the six active coelomic fluids.

All incubations of oocytes were performed in Eppendorf tubes. A 15  $\mu$ l sample of pooled oocytes from a number of different oocyte donors (as described in 3.2.1) were added to 1 ml of each chemical at each concentration, or to the negative control. All incubations were performed at 10°C in the dark (due to the photosensitive nature of some compounds). Half of the oocytes were removed after 2 hours incubation and the remainder were removed at 4 hours. After incubation all oocytes were immediately fixed as described in section 2.2.2. All oocytes were then assessed using the fluorescence assay to assess the maturational activity of each treatment.

Artificial sea water (ASW), (see appendix), was used for all chemical dilutions. Calcium-free ASW was made up according to Kuriyama *et al.* (1986) (see appendix). All chemicals were dissolved directly in ASW except for Ionophore A23187 which was first dissolved in ethanol and then subsequently diluted in ASW to give a final ethanol concentration of 1%.

The oocytes were incubated in the following treatments:

*Positive Control* (to establish that harvested oocytes had the ability to mature).

Active coelomic fluid from females 1 to 6.

*Negative Controls*

- a) ASW
- b) ASW pH 5.6 and ASW pH 7
- c) 1% ethanol in ASW (EtOH)

The ASW only, was to detect any spontaneous maturation of the oocytes. The pH manipulations were used as controls to show that a change in pH, due to dissolving chemicals in ASW, was not responsible for maturation. The pH was adjusted with 1 M HCl. All chemicals, at the highest concentrations used, had a pH of between 7.0 and 7.6, except lanthanum chloride (pH of 5.6). ASW had a pH of 7.7. The use of 1% ethanol solution was to control for possible effects of ethanol in the Ionophore A23187.

*Chemicals tested for Maturation Inducing Activity*

- a) calcium free sea water (CaFSW)
- b) tetracaine
- c) procaine
- d) propranolol
- e) lanthanum chloride
- f) oxprenolol
- g) DL-dithiothreitol
- h) serotonin
- i) ionophore A23187

The concentrations of the chemicals used were  $10^{-2}$  M,  $10^{-3}$  M and  $10^{-4}$  M, except for serotonin ( $10^{-4}$  M,  $10^{-5}$  M and  $10^{-6}$  M) and ionophore A23187 ( $10^{-5}$  M,  $10^{-6}$  M and  $10^{-7}$  M.) All chemicals were purchased from Sigma Co. except for

propranolol which was purchased from ICN Biomedicals, Inc.

## 6.3 Results

The results of the induction of oocyte maturation by specific chemicals are shown in Table 6.1. Only the positive control (coelomic fluid with CMF activity) had significant numbers of oocytes maturing. Five out of six females' coelomic fluid had CMF activity, female 6 which had no activity was therefore discarded from the experiment. In all other treatments with all chemicals at all concentrations and controls for both time periods no significant maturation of oocytes occurred.

## 6.4 Discussion

To characterise the process of oocyte maturation fully, it is essential to examine the chemical pathways involved, and specifically to look at the role of substances and chemicals already found to have an effect on maturation in other species. The aim of this experiment was to compare maturation in *Arenicola marina* to that reported previously by Meijer (1980) on *Arenicola defodiens*.

The results presented in Table 6.1 show that the oocytes from all six oocyte donors used in the experiment are competent to mature as they mature in the positive control (CMF). However, no significant numbers of oocytes undergo maturation in any of the chemicals (ionophore A23187, tetracaine, procaine, oxprenolol, propranolol, DL-dithiothreitol [DTT], serotonin and calcium free sea water) at any of the concentrations or incubation times tested. The incubation times and concentrations were all comparable to Meijer's work of 1980.

Treatment (Controls)	Incubation Time (10°C)	
	2 Hours	4 Hours
CMF	-	75 +/-3.62
ASW	0	0
ASW pH 5.6	0	0
ASW pH 7	0	0.33 +/-0.33
EtOH	0	0
<b>Treatment</b>		
CaFSW	0	0
tetracaine	0	0
procaine	0	0
propranolol	0	0
oxprenolol	0.33 +/-0.23	0
DL-dithiothreitol	0	0.11 +/-0.11
lanthanum chloride	0	0
serotonin	0.11 +/-0.11	0
ionophore A23187	0	0

Table 6.1. Response of *Arenicola marina* oocytes after incubation in different treatments; (CaFSW, calcium free artificial sea water; EtOH, ASW + 1% ethanol). For chemical treatments the response is expressed as mean percentage ( $\pm$ S.E.M.) of oocytes from 3 concentrations ( $10^{-2}$  M,  $10^{-3}$  M,  $10^{-4}$  M) except for serotonin ( $10^{-4}$  M,  $10^{-5}$  M,  $10^{-6}$  M) and ionophore A23187 ( $10^{-5}$  M,  $10^{-6}$  M,  $10^{-7}$  M). All incubations were carried out at 10°C in the dark for either 2 or 4 hours. 50 oocytes counted, three counts for each concentration. For oocytes incubated in CMF, response is expressed as mean percentage oocytes matured from incubation in 5 females' coelomic fluid.

Meijer (1980) suggested that because maturation in *Arenicola defodiens* could be induced by chemicals affecting intracellular calcium levels (tetracaine, procaine, propranolol and oxprenolol), calcium plays a key role in the maturation process in this species. From these studies in *A. marina*, elevation of intracellular calcium, specifically caused by these chemicals, does not alone induce maturation. The failure of any of these chemicals to induce maturation is in contrast to *A.*

*defodiens* (Meijer, 1980). Recently, however, the results presented here have been shown to be consistent with some work on starfish: intracellular calcium increases in starfish do occur during maturation, but they are not a requirement for maturation, or sufficient to induce maturation themselves (Kikuyama and Hiramoto, 1991). Further work to examine the exact role of calcium and whether it is essential for oocyte maturation in starfish has provided contradictory results: an increase in calcium in the nucleus, although not in the cytoplasm, is essential for maturation in starfish (Santella and Kyozyuka, 1994). Other conflicting results on the role of calcium are the lack of maturation in oocytes of starfish and *A. defodiens* when exposed to ionophore A23187 where other species' oocytes such as *Spisula solidissima* and *Pectinaria gouldii* mature in its presence (Schuetz, 1975; Anstrom and Summers, 1981). It has been suggested that in starfish the surge of calcium induced by the ionophore A23187 is too large (20 times greater than the calcium increase due to 1-MeAde) and outside the limits required to induce maturation (Moreau *et al.*, 1978).

In *Arenicola marina* oocytes, chemical induced changes in calcium levels fail to induce maturation because; a) calcium has no essential role in oocyte maturation so that changes induced in calcium concentrations are irrelevant; b) the chemicals induce too little or too much change in calcium in the oocyte to induce maturation; c) the chemicals may be acting, in this case, in a different way to their reported pharmacological action.

The fact that oocyte maturation cannot be induced by serotonin, of comparable concentrations to those that induce maturation in molluscs, suggests that the mechanism of maturation in *Arenicola marina* is not the same as certain mollusc species such as *Spisula solidissima*, *Ruditapes philippinarum* and *Dreissena polymorpha* (Hirai *et al.*, 1984; Osanai and Kuraishi, 1988; Abdelmajid *et al.*, 1993; Guerrier *et al.*, 1993; Fong *et al.*, 1994). Further work comparing molluscan maturation with *A. marina*- specifically the effect of tricyclic anti-depressants on inhibiting maturation, is described in section 5.4.



Similarly, the failure of oocytes to mature when incubated with DTT suggests that the reduction of disulphide bonds does not play a significant part in the process of maturation in *Arenicola marina*. These findings are in contradiction to the maturation process of both *A. defodiens* and starfish where DTT and another disulphide reducing agent (2,3-dimercaptoethanol) induce significant maturation (Kishimoto and Kanatani, 1973; Meijer, 1980).

The complete failure of oocytes to mature in all of the test chemicals is in contrast to the situation found not only in *Arenicola defodiens* but also starfish and many other species. These results imply that in *A. marina*, the mechanism of oocyte maturation, specifically the transduction of the CMF signal to the activation of MPF, is different to that of *A. defodiens*, and may also be different to the other more extensively studied systems described already. However, this assumes that the chemicals used in this experiment are acting in their known pharmacological manner and the concentrations used are appropriate.

## **Chapter 7**

# **The Role of Calcium in Fertilization**

## 7.1 Introduction

### 7.1.1 The Processes Involved in Fertilization

Fertilization can be regarded as a sequence of identifiable stages that form a continuum. These stages are in some ways arbitrary as the events occurring during fertilization are not discrete and may affect other events and interact with other processes. The literature pertaining to fertilization is extensive so that this introduction will only address the main points in each stage of the process. The stages of fertilization discussed here are in a general chronological order:

#### Sperm Activation

Following the completion of spermatogenesis, sperm are usually stored in an inactive form until spawning occurs. Sperm activation not only involves the acquisition of motility, but also chemokinesis (activation of the sperm by increasing the respiration and motility by chemicals released from the oocyte).

Sperm can be activated in a number of different ways, including extracellular or intracellular chemical changes as a result of hormonal stimuli. In sea urchins, sperm activation changes the internal ion concentration which results in an increase in internal pH, in turn increasing respiration rates and motility of the sperm (Nishioka and Cross, 1978; Christen *et al.*, 1981; Hansbrough and Garbers, 1981a). Sea urchin sperm can also be activated by small peptides from oocyte jelly (Hansbrough *et al.*, 1980; Hansbrough and Garbers, 1981a; Mita *et al.*, 1990).

In *Arenicola marina*, spermatozoa are of the primitive type (see Franzen, 1956). The spermatozoa within each individual morula are cytoplasmically connected to a central mass of cytoplasm called the cytophore (Pacey and Bentley, 1992b). In this state they are incapable of being spawned to the exterior of the body cavity (Howie, 1961c). Breakdown of the morulae to give free sperm is controlled by a lipid

substance. It is found within tissue extracts of males, females and immature worms at all times throughout the year, but is male specific in that the saponifiable fraction containing the substance does not cause females to spawn (Howie, 1961b; Howie, 1966). This substance was termed the Sperm Maturation Factor (SMF) (Bentley, 1985) and has putatively been identified as the fatty acid; 8,11,14-eicosatrienoic acid (only this compound and not the isomer 11,14,17-eicosatrienoic acid induces activation). However, 8,11,14-eicosatrienoic acid, does not induce activation of the sperm (sperm swimming). This is induced by increases in the internal pH which is as a result of being spawned into the sea water (Bentley, 1985; Bentley and Pacey, 1992b).

### **The Acrosome Reaction**

In species where an acrosome reaction occurs, only acrosome reacted sperm fuse with the oocyte implying that it is a vital step (Longo, 1987). Although the detail of the acrosome reaction differs between species and phyla, the reaction is essentially an event where the acrosome of the sperm undergoes a change when the spermatozoon comes into contact with the oocyte (Dan, 1967; Colwin and Colwin, 1967). It usually involves exocytosis of the acrosomal vesicle, the polymerisation of actin filaments to produce an acrosomal process, with the concomitant extension of the membrane surface (Tilney, 1975). The whole process is controlled by ionic movements (Shapiro and Eddy, 1980; Schackmann *et al.*, 1981) with evidence of the oocyte (specifically the jelly coat) initiating the acrosome reaction in starfish spermatozoa (Dale *et al.*, 1981; Ikadai and Hoshi, 1981).

### **Sperm-Oocyte Binding**

Sperm must bind with the oocyte so that the fusion of sperm and oocyte plasma membranes can occur. Sea urchin oocytes treated with proteolytic enzymes, such as trypsin, show a decrease in fertility due to a reduction in sperm-oocyte binding (Schmell *et al.*, 1977). In echinoderms, Vacquier (1980) found that a species-

specific adhesive material called bindin contained within the acrosomal vacuole mediates binding of the sperm and oocyte.

### Oocyte Activation

At sperm-oocyte contact and gamete fusion, the fertilizing sperm immediately induces electrical currents in the oocyte membrane (Dale *et al.*, 1978). This occurs when cytoplasmic continuity is established between oocyte and sperm (McCulloch and Chambers, 1992) and is the first detectable event at fertilization (Whitaker and Swann, 1993). A rapid calcium increase occurs 15 to 20 seconds later (see section 7.1.3 for detailed discussion). The increase in calcium and a slow transient increase in sodium permeability of the oocyte plasma membrane produces a transient depolarisation which is known as the fertilization potential. In the sea urchin, *Strongylocentrotus purpuratus*, the change in fertilization potential is from between -60 and -80 mV to +20 mV and persists for about one minute (Jaffe, 1976). An increase in intracellular pH also occurs which affects a number of systems including protein and DNA synthesis as well as mRNA polyadenylation and glucose-6-phosphate dehydrogenase activity (Shen, 1983). There is also an exchange of sodium and potassium with the sodium moving against a large concentration gradient (500 mM Na<sub>out</sub>: 30 mM Na<sub>in</sub>) coupled with a simultaneous increase in the potassium content of the oocyte (Girard *et al.*, 1982).

### Gamete Fusion

The fusion of a sperm with an oocyte is often restricted to a particular area of the oocyte surface. Fusion is described as a merging of two membrane domains resulting in the confluence of the protoplasmic contents of both the oocyte and sperm. However, little is known about the actual process of fusion (Longo, 1987). Colwin and Colwin (1967) stated that changes occur at the acrosomal reaction that render the sperm fusigenic with the oocyte.

During gamete fusion in marine invertebrates, a protuberance forms (the fertilization cone) from the oocyte surface at the site of fusion. This is formed by the movement of oocyte cytoplasm into the region surrounding the sperm nucleus, mitochondria and axonemal complex (Longo, 1973). The action and function of this cone is probably to help sperm incorporation, but the mechanism is unknown.

### **Polyspermy and Cortical Reaction**

There are two types of polyspermy that can occur during fertilization: in physiological polyspermy, the entry of more than one sperm into the oocyte is a common occurrence but still only one male pronucleus will associate with the female pronucleus. This situation is common in animals with large oocytes such as birds and reptiles (Longo, 1987).

Pathologic polyspermy is where the entry of more than one sperm into the oocyte will cause abnormal development even if only one male pronucleus and one female pronucleus associate to form the embryonic genome. This is the case with many invertebrates, fish and mammals. The occurrence of pathologic polyspermy is prevented by depolarisation of the oocyte plasma membrane. Although the depolarisation does block polyspermy, the temporary nature of the depolarisation requires an additional mechanism to prevent polyspermy after repolarisation of the membrane has begun. In sea urchins, as well as amphibians and fish, the "cortical granule reaction" occurs which acts as a permanent polyspermy block. In sea urchins, this involves discharge of vesicles (cortical granules) from the oocyte cortex which establishes a hard fertilization layer around the oocyte (Moser, 1939).

### **Male Pronucleus Formation**

Once the gametes have fused, the sperm nucleus must eventually fuse with the female nucleus. This requires the breakdown of the sperm nuclear envelope, chromatin dispersal and male pronuclear envelope formation. The actual times required for male pronuclear development varies from 8 min in *Arbacia*, to 50-60 min



in *Spisula* sp. (Longo, 1981). In some species, components of the sperm such as mitochondria are incorporated, although in ascidians the mitochondria are released through the tip of the sperm tail and therefore not incorporated (Lambert and Lambert, 1981). Other parts of the sperm such as the axoneme in the sea urchin *Arbacia* are incorporated and their structure is maintained until first cleavage (Longo, 1973).

### **Association of Male and Female Pronuclei.**

Once the pronuclear membranes have formed, the male and female pronuclei finally become associated with each other. From here this association may take one of two paths: a) *Ascaris* type, where male and female pronuclei break down and the chromosomes from each parent condense and then intermix on what will be the metaphase plate of the first mitotic apparatus. Oocytes of this type are always inseminated at an arrested stage of meiosis (Wilson, 1925); b) *Sea urchin* type, where the fusion of the nuclear envelopes from the male and female produces a zygote nucleus (Longo, 1973). This type of fertilization occurs in oocytes where insemination occurs after meiotic maturation. The timing of insemination therefore seems to be an important factor in determining the type of fertilization that occurs, although Wilson (1925) suggested that these two types of fertilization are extremes with intermediate forms.

## **7.1.2 The Study of Changes in Calcium at Fertilization**

### **Introduction**

Calcium is of fundamental importance as a second messenger in many systems. The rapid increase in knowledge of calcium involvement in oocyte function has been due largely to the advance in visualisation methods for calcium.

## Methods of Visualisation

### *Calcium Sensitive Dyes*

The study of calcium dynamics has been revolutionised by the development of fluorescent indicators of free calcium ion concentration which can be loaded into cells. Fluorescent dyes can be divided into 3 groups, photoproteins; ratio imaging dyes and long wavelength dyes. Photoproteins, of which aequorin is the most commonly used, emit blue-green light (470 nm) upon binding  $\text{Ca}^{2+}$  ions (Shimomura *et al.*, 1962). Ratio imaging dyes include fura-2 and indo-1. The fluorescence excitation maxima of these dyes upon binding  $\text{Ca}^{2+}$  ions, shifts to a lower wavelength with a negligible shift in the emission maximum. This allows these indicators to be used ratiometrically, making the measurement of  $\text{Ca}^{2+}$  ion concentration essentially independent of the extent of dye loading, cell thickness, leakage and photobleaching. Long wavelength dyes include fluo-3, rhodo-2, calcium green<sup>TM</sup>, calcium orange<sup>TM</sup> and calcium crimson<sup>TM</sup>. These dyes exhibit an increase in fluorescence emission intensity with little shift in wavelength upon binding to  $\text{Ca}^{2+}$  ions. They are useful for long wavelength excitation sources such as an argon laser of the confocal microscope.

### *Intracellular Introduction of Calcium Dyes into Oocytes*

Dyes can be introduced into cells by the incubation of oocytes in the permeant esterified form of the dye (with the acetoxymethyl esters being the most useful) or by the microinjection of the free acid directly into the cell. The calcium dye chosen for this study was fluo-3. The introduction into the cell by microinjection was preferred to incubation in the acetoxymethyl ester form of the dye for a number of reasons: a) Stricker *et al.* (1992) failed to obtain a utilisable signal in sea urchins with incubation in the acetoxymethyl ester form of the dye, b) the prohibitive cost of the acetoxymethyl ester form of the dye, c) the low usage of acetoxymethyl ester form of the dye in the literature compared to microinjection and d) the versatility of a

microinjection system to be used for other chemicals and treatments to be injected into oocytes.

### *Visualisation and Measurement of Calcium Changes*

The method of visualisation depends upon both the type of dye and the measurements required to be taken. Only long wavelength dyes can be used with an argon laser such as is used in the confocal microscope. Fluorometers can be used to record changes in fluorescence emission for all dyes in single wavelength mode, but to use fura-2 and indo-1 a dual wavelength fluorometer is required to make use of the ratio calibration technique.

### **7.1.3 The Calcium Wave**

The activation of an oocyte by a sperm involves a number of steps as described above. A fundamental step in activation of the oocyte is a rapid increase in intracellular calcium ions. The initiation of a calcium wave by the sperm is still not fully understood. In deuterostomes, two possible systems have been investigated. The first is that a G-protein signal transduction system may be involved that is linked to second messenger production such as inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol. The other possibility is that when a sperm and oocyte fuse, an activating messenger is injected from the sperm. Studies have, so far, failed to characterise which system is operating (Swann *et al.*, 1994).

The release of calcium at fertilization has been shown in a number of species but fundamental differences seem to be present between deuterostome and protostome species in both the origin and process of calcium release and its propagation.

### **Deuterostomes**

A calcium increase at fertilization was first observed in oocytes of a freshwater fish, the Medaka (Ridgway *et al.*, 1977; Gilkey *et al.*, 1978). In invertebrate systems much of the work has been on echinoderm oocytes, specifically on species of sea

urchin. In sea urchins, calcium release starts at the sperm-oocyte fusion site and sweeps in a regenerative fashion across the oocyte during fertilization and has been found to be both necessary and sufficient for metabolic activation (Whitaker and Steinhardt, 1985). In the sea urchin, *Lytechinus* sp., the rise in calcium induced by fertilization takes  $17 \pm 9$  seconds and falls in  $72 \pm 21$  seconds (Shen and Buck, 1993). In *Arbacia punctulata* intracellular free calcium increases from less than  $0.1 \mu\text{M}$  to  $1 \mu\text{M}$  after 23 seconds (Eisen *et al.*, 1984).

A variety of sources may contribute to the rise in calcium at fertilization but it is thought that the principal source is mobilisation of calcium associated with the endoplasmic reticulum (ER) (Jaffe, 1983; Gillot *et al.*, 1990; Terasaki and Sardet, 1991; Whitaker and Swann, 1993). The release of calcium from the ER is through two main calcium channel receptors (Berridge, 1993); i) "IP<sub>3</sub>-sensitive receptors" that are responsive to the second messenger IP<sub>3</sub>, and ii) "ryanodine receptors" that are relatively insensitive to IP<sub>3</sub> but can be stimulated by other agents, including ryanodine, caffeine and the naturally occurring ring metabolite cyclic adenosine diphosphate-ribose (Rakow and Shen, 1990; Lee, 1991; Buck *et al.*, 1992; Sardet *et al.*, 1992; Buck *et al.*, 1994).

### Protostomes

In contrast to deuterostomes, the main source of calcium for increases in calcium at fertilization in protostomes is thought to be the external medium (Jaffe, 1983). Evidence from only a few species showing calcium increases has been obtained. Oocytes of *Urechis caupo* show an increase in calcium at fertilization due to a calcium influx (Johnston and Paul, 1977). In some bivalve species (*Limarina hakodeatenisis*, *Crassostrea gigas*, and *Hiatella flaccida*) calcium increases involved an increase in the baseline level, with oscillatory increases superimposed on top of this increase. In *Mytilus edulis* the increase involved no oscillations but instead involved an increase in free calcium over the whole of the oocyte as opposed to a propagating wave-like event (Deguchi and Osanai, 1994).

Recently, work on the annelid *Chaetopterus pergamentaceus* has suggested calcium transients at fertilization have more in common with deuterostomes than previously thought. In this protostome, fertilization initiates multiple brief calcium transients, the first is not propagated but the following transients propagate from pole to pole interspersed with non propagated pulses. These transients, however, did traverse the oocyte at a much faster rate ( $30 \mu\text{m}.\text{sec}^{-1}$ ) than seen in deuterostomes ( $5\text{--}10 \mu\text{m}.\text{sec}^{-1}$ ) (Eckberg and Miller, 1995).

Evidence suggests that, in bivalves at least, the use of external calcium rather than internal stores is not the sole store for calcium increases; removal of external calcium does not inhibit calcium increases which implies that internal stores are also important in calcium increases at fertilization (Deguchi and Osanai, 1994). There is also evidence to suggest in deuterostomes, specifically from studies of sea urchin species, that the supposition that internal stores are the important sources of calcium may also be erroneous. Gillot *et al.*, (1993) working on *Lytechinus pictus*, found that a small spherically symmetrical increase in calcium is blocked by removing external calcium or by the addition of the voltage-sensitive calcium channel blocker, diltiazem.

### Action of the Calcium Wave

The action of the increases in calcium and continued calcium transients through development is to control the cell cycle progression. In sea urchins post-translational and translational regulation of MPF is controlled by calcium transients. These transients trigger phosphorylation and protein synthesis, specifically the synthesis of cyclin and phosphorylation of pp34, at the start of mitosis. A transient at the exit from mitosis causes the destruction of phosphorylated cyclin (Whitaker and Patel, 1990).

In the bivalve, *Mytilus edulis*, *in vitro* incubation of oocytes with emetine or 6-dimethylaminopurine (6-DMAP), which inhibit protein synthesis and protein phosphorylation respectively induce maturation (Neant *et al.*, 1994). These authors concluded that release from metaphase I arrest in *M. edulis*, triggered by fertilization,

is induced by the rapid destruction of a set of continuously synthesised proteins. This is accompanied by decreased H1 histone kinase activity. Release from metaphase I arrest in *Patella vulgata* oocytes (triggered by fertilization) could also be induced *in vitro* by protein inhibition (Neant and Guerrier, 1988). Further work by Colas *et al.* (1993) has shown that a transient inhibition of translation in activation of cyclin proteolysis is involved in the release of oocytes from this second block. Abdelmajid *et al.* (1993) also found in *M. edulis* and *Ruditapes philippinarum* that calmodulin antagonists reversibly block release from metaphase I, but do not have any effect on emetine (which inhibits protein synthesis) induced release.

In this study a microinjection system was set up to look at any changes (if present) in free calcium levels at fertilization within oocytes of *Arenicola marina* and *Nereis virens*. To calibrate the system and to ensure that it worked effectively, changes in calcium at fertilization were measured with oocytes of the starfish *Asterias rubens* and the sea urchin *Echinus esculentus*. Intracellular calcium changes have already been documented in a number of other species of starfish and sea urchins (for review see Whitaker and Swann, 1993).

## 7.2 Materials and Methods

### 7.2.1 Collection and Maintenance of Animals

Mature *Nereis virens* were kindly donated by Seabait Ltd, Lynemouth, Northumberland. These were transported back to the laboratory individually in plastic containers with a small amount of sea water and washed gravel. In the laboratory animals were kept separately with a small amount of gravel in plastic boxes with 0.5 cm diameter holes drilled in all sides. The boxes were then placed in a tank with running sea water at 5°C with ambient illumination. This provided worms with a constant supply of clean water but, at the same time, kept them separate from other



worms to prevent damage and escape. Mature specimens of *Arenicola marina* were obtained and maintained as described in section 2.2.1.

Mature specimens of *Asterias rubens* and *Echinus esculentus* were collected from various sites around the Scottish Coast using snorkelling apparatus. They were maintained in aquaria at ambient temperatures and illumination until required for use.

### **7.2.2 Removal of Gametes and Assessment of Maturity**

*Nereis virens* were held down on a paper towel to remove excess water and reduce movement and samples of coelomic contents were removed using a 1 ml syringe with a 21 g hypodermic needle inserted between the segments in the posterior region of the worm. Coelomic fluid containing gametes was slowly withdrawn and examined under a compound light microscope with x10 objective. Maturity of males was assessed by estimating the relative proportion of active sperm when diluted in sea water. For females, maturity was assessed by a fertilization test (see section 7.2.3).

Assessment of maturity in *Arenicola marina* was carried out using a 1 ml syringe with a 19 g hypodermic needle inserted through the body wall where the trunk meets the tail. A small sample of coelomic fluid, containing gametes, was slowly withdrawn and examined under a compound light microscope with x10 objective. The maturity of males was assessed by estimating the number of spermatocyte clusters and morulae under a compound light microscope using a x10 objective. The diameters of oocytes taken from females were compared to fully grown oocyte diameters.

Gonads containing mature gametes of *Asterias rubens* were removed by dissection of an arm and were examined for fully developed oocytes and sperm under a compound light microscope with x10 objective. Once dissected, gonads could be maintained for 24 hours at 4°C before use.

### 7.2.3 Induction of Spawning and Obtaining Mature Oocytes

#### *Arenicola marina*

In all experiments, except where stated otherwise, oocytes and sperm from *Arenicola marina* were obtained from individuals that had been induced to spawn by injection of homogenised prostomium, equivalent to one male prostomium per male or 1 equivalent female prostomium per individual female. Fertilizations with gametes from individuals that had been induced to spawn were successful and produced motile trochophore larvae. It may be assumed that fertilization and development are the same in embryos obtained from induced spawnings as embryos from naturally spawned gametes, although see section 8.4.2 for further discussion of the validity of this assumption.

#### *Nereis virens*

All experiments with *Nereis virens* were carried out with gametes that had been removed directly from individual animals as described above as no natural spawning by individuals was observed in the laboratory.

Individual males were checked for active sperm by removing a small sample of coelomic fluid and diluting it in sea water, and then checking for active sperm by light microscopy. In some cases sperm were still in the tetrad form so these males were left to mature further. Only individuals with clearly active sperm were used for fertilization.

Although all females had fully grown oocytes (180  $\mu\text{m}$ ), not all individuals had oocytes that would fertilize. Females' oocytes were tested for their ability to fertilize by removing a sample of oocytes and adding active sperm to them. After 10 minutes oocytes were observed and the presence of the oocyte jelly signified that oocytes were fertilizable.

*Asterias rubens*

Mature oocytes and active sperm were obtained by incubating dissected gonads in sea water containing  $10^{-4}$  M 1-MeAde for approximately 1 hour. Mature oocytes (checked by the absence of a GV) and active sperm were released from the gonads and were stored at 4°C and used within 24 hours.

*Echinus esculentus*

Spawning was induced by the injection of approximately 5 ml of 0.5 M KCl, through the soft tissue surrounding the mouth, into each sea urchin. Each injected urchin was then inverted and placed in a beaker of sea water and subsequently spawned mature gametes into the beaker. Once spawned gametes could be maintained for 24 hours at 4°C.

**7.2.4 The Microinjection Procedure****Preparative Techniques***Preparation of Coverslips*

All coverslips (22 x 22 mm, thickness No. 1) were soaked in a 1% solution of Neutacon (Decon Laboratories Ltd.) for 1-2 hours. They were then rinsed under running water for 24 hours before storage in 75% ethanol. Coverslips were then placed in poly-L-lysine solution (Sigma Co.) for 5 minutes, drained and then dried for 1 hour at 60°C.

*Construction of Microinjection Coverslip*

Individual microinjection slides were made using the pre-treated coverslips. A small piece of coverslip (11 mm x 3 mm) was cut using a diamond pencil and placed on two strips of masking tape (the spacer) positioned on a full coverslip approximately 1-2 mm from one edge. The piece of coverslip was fixed to the spacer using melted paraffin wax dropped onto the top of the coverslip piece. The completed

microinjection coverslips could then be stored in a dust free environment until required for use.

### *Microelectrode Pulling*

Microinjection electrodes were pulled using GC150F-10 filamented glass capillary tubes (Clark Electromedical Instruments) on a vertical microelectrode puller (Model 720, David Kopf Instruments, USA.). The heat and solenoid were adjustable, but 10.4 heat and a solenoid setting of 0 were found to give the most consistent results.

### *Initial Assembly of Injection Chamber*

The injection chamber was assembled prior to experimentation and consisted of 3 parts:

1. A support slide machined from aluminium of 7.5 cm x 2.5 cm x 0.45 cm. Figure 7.1 shows a diagrammatic representation of the support slide. Silicon grease was applied to both sides of the support slide;
2. The inverted microinjection coverslip was attached to the support slide;
3. To complete the chamber a bottom coverslip was attached to the support slide with silicon grease.

### *Microinjection Apparatus*

Microelectrodes were held in a holder made of perspex mounted on a micromanipulator (Prior, England). To deliver a pulse of pressure to the microelectrode, a length of 4 mm diameter pneumatic tubing connected the electrode holder to a manual pump spray through a solenoid valve (24V dc, Radio spares). Pulses of pressure were delivered at a frequency of approximately 1 Hz controlled by a SS-555 chip time base counter with potentiometer (Radiospares). Pulse duration could be varied from 10 to 50 msec. An in-line flow regulator before the solenoid valve controlled output of pressure. Injections were performed under an Olympus compound microscope with a x40 objective.

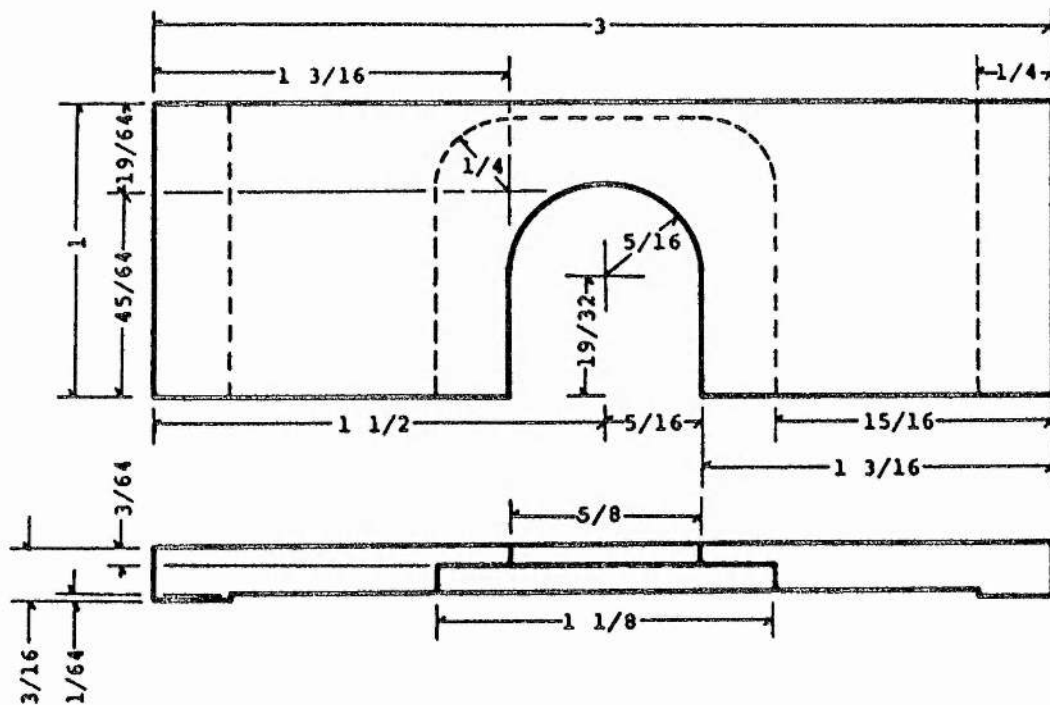


Fig.7.1. Schematic diagram of the support slide for the microinjection apparatus with specifications in inches (after Begg and Ellis, 1979).

*Dye preparation and Loading Microelectrodes*

Fluo-3 pentapotassium salt (Molecular Probes Inc.) was dissolved in de-ionised water ( $10^{-2}$  M) and frozen at  $-20^{\circ}\text{C}$  in aliquots until required. Once defrosted, the dye was diluted in intracellular buffer to give a final concentration of  $5 \times 10^{-3}$  M. The intracellular buffer consisted of 0.5 M KCl, 20 mM PIPES and 100  $\mu\text{M}$  EGTA with the pH adjusted to 6.8 with 1 M HCl (see appendix). All microelectrodes were backfilled by capillary action.

*Calibration of Microinjection System*

The system was calibrated by adjustment of the in-line flow regulator and the number of pumps on the pressure spray. Injection under mineral oil onto coverslips coated with Replicote (Sigma Co.) to produce spheres which could then be measured using a calibrated eye piece graticule to obtain a measure of volume. Injection of between 1 pl and 5 pl was consistent with approximately 0.1% of oocyte volume.

*Loading Oocytes into Microinjection Chamber and Final Assembly*

A dense suspension of oocytes was sucked into the end of a 20  $\mu\text{l}$  microcapillary tube. The end of this tube was then placed on the edge of the coverslip piece, releasing the oocytes in a line along the chamber opening. Oocytes were drawn into the chamber by touching a piece of tissue to one end of the chamber. As soon as oocytes were loaded into the chamber the coverslip was inverted and attached to the support chamber with silicon grease. The space between the two coverslips was then filled with TFSW. Oocytes were checked before proceeding with injection to make sure they were not too flattened.

**Injection Procedure**

Figure 7.2 shows a schematic diagram of the completed microinjection chamber. The injection chamber was screwed down onto a block of perspex to give enough clearance between the electrode holder and the microscope stage. The



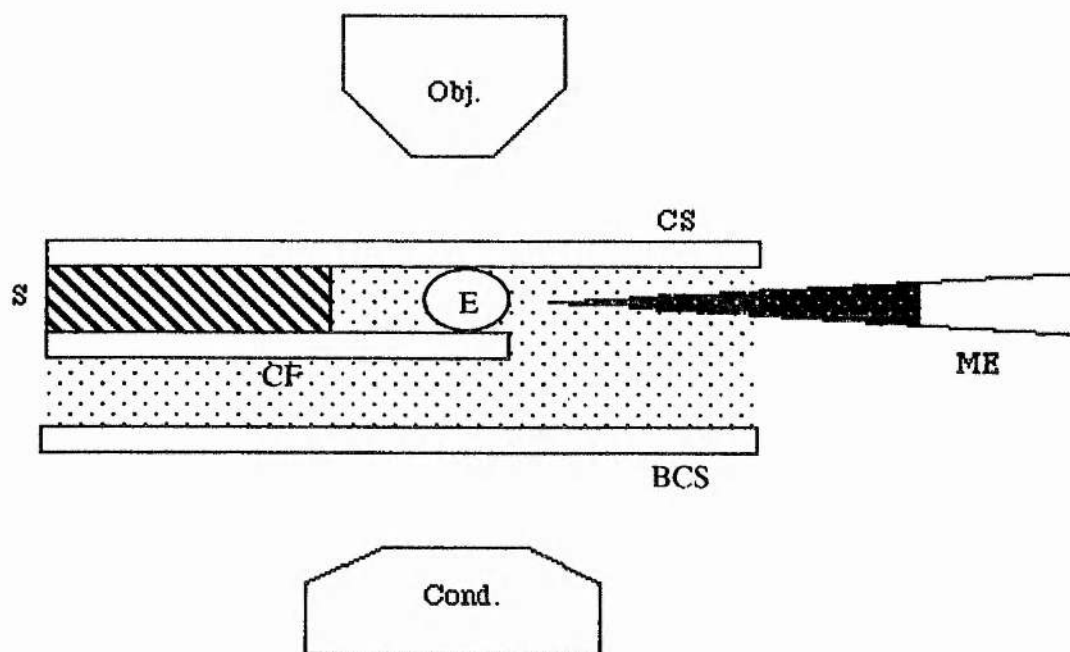


Fig. 7.2. Cross-sectional schematic of the injection slide. A cutaway view of the injection slide schematically depicts the relationship between egg, microinjection chamber, loaded microelectrode and microscope just prior to an injection. Cross section is in the plane defined by the microscope's optical axis and the long axis of the microelectrode. Obj., objective; Cond., condenser; BCS, bottom coverslip; CF, coverslip fragment; E, egg; S, spacer; CS, top coverslip; ME, loaded microelectrode (from Kiehart, 1982).

electrode was brought into focus first and then the injection chamber was also brought into focus next to the electrode. The electrode was then moved down out of focus and the chamber brought towards the electrode until the electrode was immersed in the water of the chamber. The electrode was then refocused with the x10 objective. Magnification was then increased to x40 and the tip of the electrode and the edge of the coverslip fragment were brought into focus. The tip was then broken by gently touching the electrode tip on the edge of the coverslip fragment. The external diameter of the tip, after breakage, was approximately 1-3  $\mu\text{m}$ . This was sufficient to prevent blockage but not too large to damage the oocytes.

To inject an oocyte, the oocyte had to be moved into the same plane of focus with the tip of the electrode. The oocyte was then moved onto the tip of the electrode piercing the outer membranes until the end of the tip was in the centre of the oocyte. A pulse was then given to expel fluo-3 into the oocyte. This could be seen by the displacement of the cytoplasm and the presence of the fluo-3 before it dispersed through the oocyte. Once expulsion of the dye was complete the oocyte was removed from the tip and another oocyte was lined up for injection.

### **Observation of Injected Oocytes**

Once all the oocytes were injected, the chamber was placed in a cold, sealed container to prevent evaporation during transportation to the confocal microscope. Oocytes were visualised with bright field optics to get the correct focus (the centre of the oocyte) before being observed using confocal microscopy. The confocal laser scanning microscope used was as described in section 2.2.3.

### **Fertilization of Oocytes on the Confocal System**

Once the chamber was placed on the stage and correctly focused, an image of the oocytes was taken, before sperm was added, as a control. The time series was then set up and then a drop of diluted sperm ( $10^4$  sperm.ml<sup>-1</sup>) would be added to the sea water and the time series started.

## Confocal Settings

Scanning was initiated immediately after the addition of sperm to the chamber. The interval between each scan was 10 seconds, and continued for 5 minutes. Each picture was stored directly on 5<sup>1</sup>/<sub>4</sub>" optical disks (Phillips). To visualise any changes in fluorescence intensity, after each completed run, each image was pseudocoloured using the GEOG facility of the confocal. Copies of data were downloaded using a colour video printer (Sony VP5000) onto colour printer paper.

## Verification of the Microinjection System

Before proceeding with the microinjection experiments, it was necessary to investigate the effect of fertilizing oocytes within the chamber and the effect of injecting oocytes on their fertilization and development. To assess the effects of the chamber, oocytes were placed in the chamber and then fertilized and incubated at 10°C for 5 hours and their development assessed.

To assess whether the microinjection of oocytes affected their fertilizability and subsequent development, oocytes were injected with intracellular buffer only and then fertilized and incubated for 5 hours at 10°C.

## 7.3 Results

Once the microinjection chamber and apparatus had been assembled the initial aim was to get normally developing embryos after fertilization within the chamber. Figure 7.3 shows a 4 cell stage *Echinus esculentus* embryo that was fertilized and then incubated for 5 hours within the injection chamber at 10°C. This is representative of oocytes fertilized within the chamber and confirms that fertilization and initial development within the chamber is therefore apparently normal.

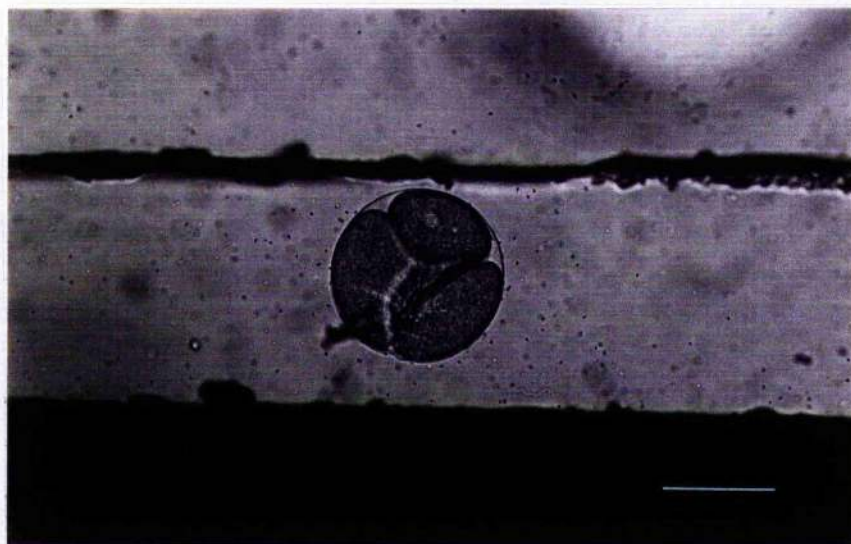


Fig. 7.3 A sea urchin embryo, (*Echinus esculentus*) fertilized in the microinjection chamber. The embryo has reached the 4 cell stage after 5 hours incubation within the chamber at 10°C. Scale bar=100  $\mu$ m.

The next step was the injection procedure itself: Would the injection of intracellular buffer adversely affect the fertilization and/or development of an oocyte? Preliminary studies suggest that in some experiments with *Asterias rubens*, injected oocytes fail to fertilize or develop with intracellular buffer. However, other oocytes injected in the same chamber and with the same microelectrode did fertilize and develop, although none of the oocytes develop properly. In experiments with *Nereis virens* some oocytes also fertilize after injection (confirmed by the raising of the jelly coat), but again other oocytes fail to fertilize. The fertilized oocytes also fail to develop normally; some did not cleave at all, while some cleaved abnormally.

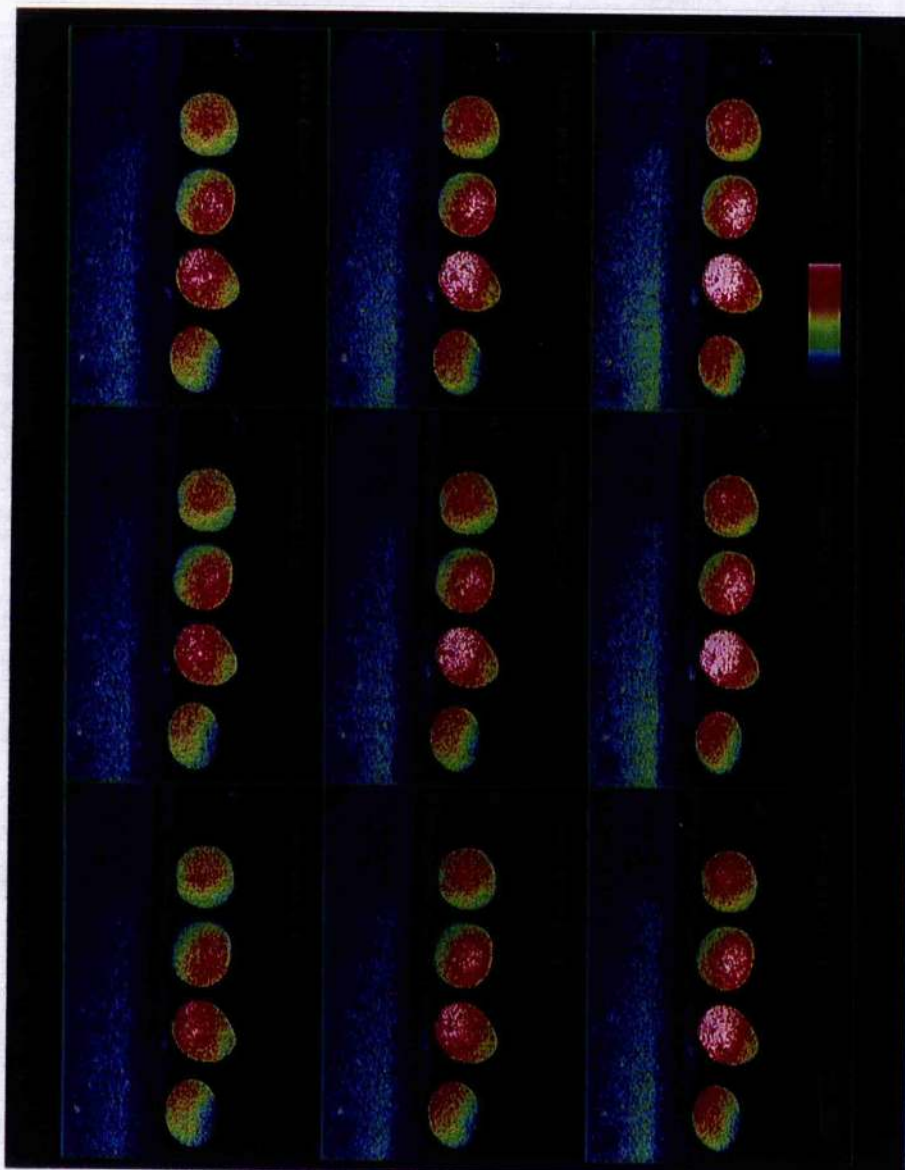
The injection of fluo-3 into oocytes and their subsequent fertilization and visualisation under the confocal microscope was found to be problematic (see discussion). A number of attempts were made using different concentrations of fluo-3 and different experimental animals. Results presented in Figure 7.4 are of four *Asterias rubens* oocytes injected with fluo-3 in intracellular buffer ( $10^{-3}$  M). Figure 7.4 shows nine sequential images (A-I). Image A was taken just prior to the addition of sperm, the other images were taken at 30, 70, 110, 150, 190, 230, 270 and 290 seconds after the addition of sperm. Throughout the course of the time series there is a gradual increase in the levels of fluorescent intensities in all of the oocytes (blue signifies low and reds and whites signifies high fluorescent intensities). In all the oocytes there is just a general increase from the centre of the oocyte which spreads to the periphery and no wave-like changes.

## 7.4 Discussion

Results presented here suggest that the fertilization and subsequent development of oocytes within the microinjection chamber are normal at least during the early cleavage stages. Fertilization and developmental problems occurred after the injection of the oocyte. The limited time available to research this problem, however,

Fig. 7.4. Time-lapse confocal microscopy of four *Asterias rubens* oocytes loaded with fluo-3 at  $10^{-3}$  M (dissolved in intra-cellular buffer) by microinjection. Pseudocoloured images depict low fluorescent intensities as blues, and whites as high fluorescent intensities. Oocytes were scanned through the centre of the oocyte prior to the addition of sperm (Image A, T=0 sec) and then every 10 seconds for 5 minutes after the addition of sperm of concentration  $10^4$  sperm.ml<sup>-1</sup>. Times for each image are as stated: images B, C, D, E, F, G were scanned at 30, 70, 110, 150, 190, 230, 270, 290 seconds respectively after the addition of sperm. Scale bar=200  $\mu$ m.





resulted in an incomplete examination of the reasons why some oocytes fail to develop. In some cases the microinjection electrode tip was too wide resulting in the leakage of cytoplasm from the oocyte when the tip was retracted. The injection procedure appeared normal in other oocytes, although oocytes still failed to develop. In these cases other unknown factors may have affected fertilization and development such as high temperatures during the injection and confocal analysis periods (22°C approximately).

The results presented in Figure 7.4 initially suggest that the changes in fluorescent intensities are a result of increases in internal calcium concentration within each oocyte. These increases in calcium concentration are a result of the addition of the sperm and subsequent fertilization of the oocytes. However, detailed examination of the results suggest that other factors, unrelated to internal calcium changes, may be responsible for the changes in the fluorescent intensities.

The most likely explanation for the increases in fluorescent intensities within the oocytes is that they are an artefact of the microscopical examination. In the top left corner of each image there is an area of fluorescent intensity that is not related to any oocyte. This area of intensity increases at approximately the same rate as the increases within the oocyte through each image. This area of increased intensity suggests that the changes in fluorescent intensity are a result of changes within the whole chamber. This may be induced by slight changes in the level of the focal plane which, in each subsequent image, focuses on an area of oocyte with a higher fluorescent intensity. A leakage of dye in the chamber might also have an effect on fluorescent intensities within an oocyte. Leaked dye may interfere with the injected dye within the oocyte. This could give a false image because of the calcium in the sea water medium causing the leaked dye to fluoresce. The hypothesis that the change in fluorescent intensities is as a result of artefacts, is supported by results from other studies of calcium waves in echinoderms and other marine invertebrates.

Studies in other deuterostomes have shown that the calcium increases follow a general wave pattern starting from the point of sperm entry (Jaffe, 1983; Eisen *et al.*, 1984; Yoshimoto *et al.*, 1987; Hamaguchi and Hamaguchi 1990; McCulloch and Chambers, 1991; Stricker *et al.*, 1994; Stricker, 1995). In the results presented in Figure 7.4 none of the oocytes have increases with wave-like characteristics that include a clear front and a following increase behind this wave front. A number of studies in starfish have also shown the wave to propagate at speeds ranging from 4-7 to 10  $\mu\text{m}.\text{sec}^{-1}$  (Stricker *et al.*, 1994; Stricker, 1995). If a similar rate of propagation was present in these oocytes then the increase would have propagated across the whole of the oocyte within approximately 50 seconds even at the slowest rate. None of the oocytes in Figure 7.4 show propagation rates of this speed therefore supporting the hypothesis that the increases recorded in these oocytes are not fertilization induced calcium changes in origin.

Figure 7.4 also shows that the increases in fluorescent intensities can be visualised after approximately 30 seconds and continue at a steady rate until image collection ceased at 5 minutes post-fertilization. In other species of starfish the wave begins 50-150 seconds after addition of sperm (Stricker *et al.*, 1994; Stricker, 1995), although in sea urchins the wave begins within 13-23 seconds (Eisen and Reynolds, 1985; Shen and Buck, 1993). Once initiated the wave is propagated over the whole of the oocyte within approximately 3 minutes (Stricker, 1995). Once propagation was complete the elevated levels were maintained for 10-30 minutes (Poenie *et al.*, 1985; Stricker *et al.*, 1992; Gillot and Whitaker, 1993, Shen and Buck, 1993; Stricker, 1995). None of these characteristic features were present in the oocytes in Figure 7.4. This further supports the supposition that the change in fluorescent intensity was not calcium related, but was an artefact of the experimental protocol or method of visualisation.

## **Chapter 8**

### **Post-fertilization Development and Microtubule Distribution**

## 8.1 Introduction

Mature oocytes of *Arenicola marina* are fertilized at the metaphase I stage of meiosis as described in section 1.3.2. Upon fertilization, a fertilization membrane is raised and the oocyte also becomes nearly spherical (Newell, 1948). Within 2-2.5 hours two polar bodies are produced, which lie within the egg membrane. This is followed by cleavage which is of the spiral type as is typical for a protostome. *A. defodiens* is also fertilized at the metaphase I stage of meiosis (Meijer, 1980).

In contrast, fertilization in *Nereis virens* occurs at the prophase stage of the first meiotic division as described in section 1.3.3. Upon penetration of the oocyte by the sperm, large cortical alveoli in the peripheral cytoplasm of the oocyte breakdown releasing jelly in precursor form into the perivitelline space. The jelly is then released through pores left by the retraction of the microvilli (Bass and Brafield, 1972). The jelly coat may reach a thickness of 120  $\mu\text{m}$  with a gap of 0.3  $\mu\text{m}$  between the jelly and the fertilization membrane and all the jelly is exuded within 30 minutes of fertilization (Bass and Brafield, 1972). Not all oocyte populations produce jelly coats with some females producing embryos of which only 3% have jelly coats. It is uncertain whether this is genetic but subsequent development does not seem to be affected by a lack of jelly coat (Bass and Brafield, 1972). Cleavage is spiral and first cleavage occurs after approximately 3 hours, although development is asynchronous (Bass and Brafield, 1972).

The post-fertilization development of *Arenicola marina* and *Nereis virens* through to blastula stage has been studied using light microscopy (Blegvad, 1923; Newell, 1948; Bass and Brafield, 1972). However, nothing is known of the microtubule structures associated with the development of these species. With respect to other marine invertebrates, studies of microtubule structures through fertilization and development to blastula have concentrated mainly on the echinoderms, specifically the sea urchins and starfish. Although there has been some more recent work on other



phyla, no work of a comparable type has been attempted in either *Arenicola* sp. or *Nereis virens*.

### 8.1.1 Microtubule Labelling in Other Species

There have been two approaches to the examination and characterisation of microtubule structures in fertilized oocytes. The first is to examine isolated microtubule structures *in vitro*, the second is the examination of microtubule structures in intact cells.

In the sea urchin *Stronglyocentrotus purpuratus*, extraction of isolated microtubules and their subsequent polymerisation and depolymerisation *in vitro* has enabled rates of growth and destruction of spindles to be followed and has shown that spindle tubulin can assemble at 37°C in the absence of detectable microtubule associated proteins (MAPs). This assembly is at a low critical concentration for polymerisation but at physiological temperatures is maintained at a steady state. No other type of tubulin has been reported to polymerise at such a low temperature (Keller and Rebhun, 1982).

The extraction of microtubules in the surf clam *Spisula solidissima* has also enabled workers to characterise specific proteins associated with spindle microtubules (Murphy, 1980). In addition, components of the centrospheres (equivalent in function to the centrosomes, but larger and the focus of microtubule organisation) have been identified in the sea urchin *Stronglyocentrotus purpuratus* as polypeptides (Kuriyama and Borisy, 1985).

Although the use of isolated spindles has provided important information on mechanisms of spindle formation and the identification of molecular components of the mitotic spindle, this method fails to show a holistic view of microtubule development in conjunction with other events. The study of microtubules in intact cells by whole fixation or microinjection techniques provides the closest representation



to the *in vivo* microtubule cytoskeleton and how this relates to other features of development.

The labelling of mitotic spindles with antitubulin staining in whole invertebrate oocytes was first successfully attempted in sea urchin oocytes by Harris *et al.*, (1980). These authors followed the distribution of tubulin-containing structures from fertilization through to first cleavage in the sea urchin, *Strongylocentrotus purpuratus*. Further studies using the same technique, although different fixation protocols, have shown the microtubule movements occurring at specific time points from fertilization can be characterised and followed in other species of sea urchin such as *Lytechinus pictus*. Although the results obtained were similar, the different fixation protocols improved the resolution and enabled other structures to be recorded (Hollenbeck and Cande, 1985). Similar studies with oocytes of the sand dollar, *Clypeaster japonicus*, and *Spisula solidissima* have provided time series information. In *S. solidissima* comparisons of microtubule structures were made with parthenogenetically activated oocytes and normally fertilized oocytes (Kuriyama *et al.*, 1986). In *C. japonicus* the injection of heterogeneous fluorescent labelled tubulin at different stages after fertilization suggested that heterogeneous tubulin undergoes spatial and stage specific regulation of assembly-disassembly in the same manner as the sand dollars own tubulin (Hamaguchi *et al.*, 1985).

The use of microtubule inhibitors and other specific reaction inhibitors in conjunction with antitubulin labelling have also shown the importance of specific molecules and reaction pathways in regulating development and microtubule growth. In sea urchin embryos, the use of the protein phosphorylation inhibitor 6-dimethylaminopurine (6-DMAP) has shown that protein phosphorylation is required for sperm aster microtubule growth and migration, but not for cortical microtubule polymerisation (Dufresne *et al.*, 1991). Bestor and Schatten (1981) used antitubulin labelling and the microtubule inhibitors griseofulvin and colchicine to follow microtubules during pronuclear movements of sea urchin fertilization. They found

that pronuclear movements and the assembly of detectable microtubules during these movements are sensitive to the inhibitors. These pronuclear movements result from the actions of the sperm aster representing microtubule mediated motility.

Ooplasmic movements have also been shown to be mediated by microtubules and microfilaments which comprise the majority of the cytoskeleton. In the ascidian *Molgula occidentalis*, studies have shown that ooplasmic segregation after fertilization is mediated by these two systems. At fertilization, in this species, the egg cortex contracts (phase 1) and then mitochondria migrate as the myoplasmic crescent develops (phase 2). The second phase is inhibited by the microtubule inhibitors colcemid and nocodazole and the first phase is inhibited by cytochalsin E which inhibits microfilaments. These results show that microtubules and microfilaments operate independently from each other but both are required to complete ooplasmic segregation (Sawada and Schatten, 1989).

This chapter documents the changes in microtubules and chromosome positions from fertilization through to blastula stage in *Arenicola marina*, *A. defodiens* and *Nereis virens*. Although a near-complete time series was achieved for *A. marina*, problems encountered with the fixation and labelling procedure for *N. virens* resulted in an incomplete time series. Changes in microtubular structure were also followed through fertilization in *A. defodiens* to compare and contrast development with *A. marina*, although a lack of specimens also resulted in an incomplete time series.

Section 8.2.2 of this chapter looks at the effect that different methods of oocyte maturation in *Arenicola marina*, as described in section 2.2.4, have on the speed of development. In section 2.4, different methods of maturation and their effects on the meiotic metaphase spindle have already been addressed. Section 8.3.3 is a continuation of this experiment.

## 8.2 Materials and Methods

### 8.2.1 Collection and Maintenance of Animals

Mature specimens of *Arenicola marina* and *Nereis virens* were obtained and maintained as described in section 2.2.1. and 3.2.1 respectively. Mature specimens of *A. defodiens* were collected by P. S. Cadman from a number of sites in Northumberland using an Alvey bait pump which sucks the worm out of its burrow. They were maintained as described for *A. marina*.

### 8.2.2 Removal of Gametes and Assessment of Maturity

For *Arenicola marina* and *Nereis virens*, removal of gametes and assessment of maturity was as described in section 3.2.1. For *A. defodiens* removal of gametes and assessment of maturity was as described for *A. marina*

### 8.2.3 Fertilization and Culture of Embryos

#### *Arenicola marina* and *Arenicola defodiens*

Fertilization in *Arenicola marina* and *A. defodiens* was obtained with oocytes and sperm from individuals that had been induced to spawn by injection of 1 male or 1 female prostomium equivalent into each male and female respectively, as described in section 3.2.3.

After release of the gametes into 150 ml of sea water, oocytes were removed and placed in 100 ml of TFSW in a crystallising dish. In cases where more than one female was induced to spawn all the oocytes were pooled unless specific individual fertilization was required. The same process was repeated for males except that haemocytometer counts of sperm were taken to give a final concentration of  $10^4$  sperm.ml<sup>-1</sup> when added to the oocyte suspension. Oocytes and sperm were incubated for 10 minutes at 10°C before changing the water and then maintained at 10°C under

ambient illumination. To prevent the aggregation of embryos which could otherwise affect development of the embryos, the water was stirred periodically.

### *Nereis virens*

All fertilizations with *Nereis virens* were carried out with gametes that had been removed directly from individual animals as described in section 7.2.1 as no natural spawning by individuals was observed in the laboratory.

Individual males were checked for active sperm by removing a small sample and diluting in sea water and then checking for active sperm by light microscopy. In some cases sperm was still in the tetrad form and in these cases individuals were left to mature further. Only individuals with clearly active sperm were used for fertilizations. In some females, fully grown oocytes (180  $\mu\text{m}$ ) were not always fertilizable. Oocytes were therefore tested for their ability to fertilize by removing a sample of oocytes and adding active sperm to them. After 10 minutes oocytes were observed and the presence of the egg jelly signified that oocytes were fertilizable.

In males with active sperm and females with fertilizable oocytes gametes were removed as described in section 3.2.1 and used for fertilization. Approximately 2 ml of oocytes were placed in 100 ml TFSW and sperm was added to give a final concentration of  $10^4$  sperm.ml<sup>-1</sup>. Incubation was at 10°C under ambient illumination and the water was changed after 10 minutes to remove excess sperm. An aquarium air pump and airstone were used in conjunction with periodic stirring to prevent aggregations of embryos and similar problems occurring as for *Arenicola marina* embryos.

## **8.2.4 Sequential Observations of Post-fertilization Development using Microtubule and Chromosomal Labelling**

Oocytes of *Arenicola marina*, *A. defodiens* and *Nereis virens* were fertilized as described above. Embryos of all three species were incubated at 10°C under ambient

illumination. Fixation, labelling and visualisation of chromosomes and microtubules for specific stages of *Arenicola marina* was as described in section 2.2. Fixation of *A. defodiens* was as described for *A. marina*.

The fixation of *Nereis virens* embryos using the fixation protocol that had been used for *Arenicola sp.* failed because of the inability of the fixative to penetrate the large jelly coat produced at fertilization, which surrounds the embryos. To overcome this problem either the jelly coat needed to be removed prior to fixation or, alternatively a stronger or more penetrative fixative could be employed to penetrate the jelly coat. After failure to obtain labelling, a number of different techniques developed for removal of fertilization membranes or to prevent them forming in sea urchin embryos were evaluated. The most successful method is given below:

### **Removal of Jelly Coat**

One millilitre of embryos in sea water were placed in a watch glass with 1 ml of Jelly Coat Removal solution (see appendix) and left for 1 minute at room temperature. The embryo solution was then passed 10 times through a 180  $\mu\text{m}$  mesh with a 2.5 ml disposable syringe. This treatment removed the majority of the jelly coat without obvious damage to the embryo.

### **Fixation and Rehydration of Embryos and Oocytes**

The embryos were centrifuged gently (approximately 500 rpm) in an Eppendorf tube for 10 seconds, after which 95% of the Jelly Coat Removal/sea water solution was removed and replaced by 1 ml of -20°C Dent's fixative (see appendix) and then incubated at -20°C for 30 minutes. During the incubation period, Eppendorf tubes were periodically shaken to prevent aggregation of the embryos.

A rehydration series (as described below) was performed at room temperature with 10 minutes for each step. After each stage, 95% of each solution was removed

and replaced with the next solution in the series until the embryos were rehydrated in PBS buffer (no Triton X-100) (see appendix).

100% methanol

70% methanol 30 % PBS buffer (no Triton X-100)

50% methanol 50 % PBS buffer (no Triton X-100)

30% methanol 70 % PBS buffer (no Triton X-100)

100% PBS buffer (no Triton X-100)

Once rehydrated, the embryos and were processed for immunofluorescence and visualised as described in section 2.2.3 and using a Zeiss DF20. Photographs were taken with Kodak monochrome TMY 400 ASA. In addition the mitotic stage of 50 oocytes were assessed at each time interval.

### **8.2.5 Effects of Maturation Method on Development Rates in *Arenicola marina***

Oocytes were matured by the three different methods described in section 2.2.4: *in vitro* incubation in CMF; induction of spawning by injection of homogenised prostomia; and obtaining mature oocytes by natural spawning. After incubation at 10°C for 5 hours, embryos were fixed and labelled as described in section 2.2.3.

Embryos were examined as described in section 2.2.3. Three replicate counts of 50 embryos were scored for each developmental stage. Embryos were classed according to which mitotic cleavage and which stage of the mitotic process they had reached. Embryos were classed as interphase/prophase, metaphase, anaphase, and telophase of each mitotic cycle.



## 8.3 Results

### 8.3.1 Sequential Observations of Post-Fertilization using Microtubule and Chromosome Labelling: a Time Series

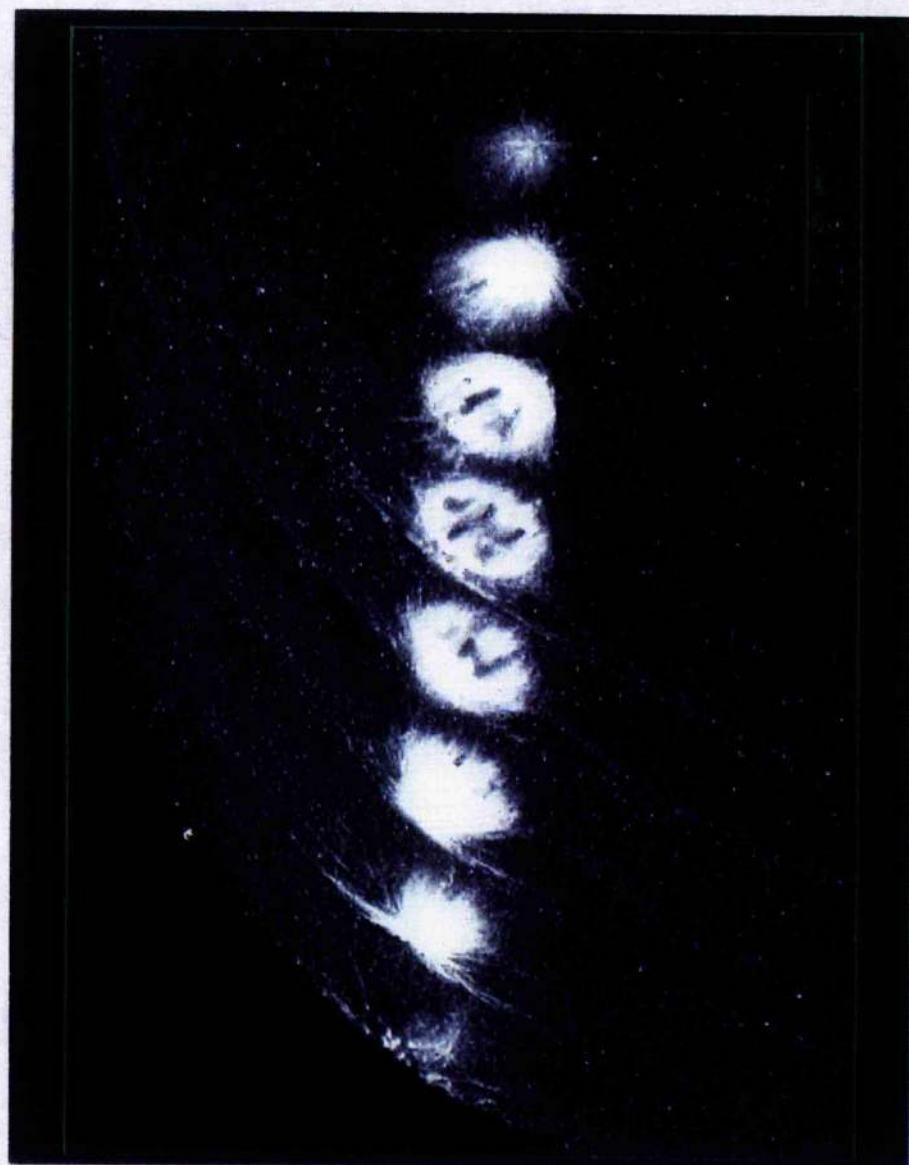
#### *Arenicola marina*

Figures 8.1 to 8.13 show the changes in microtubule distribution and corresponding chromosomal arrangements from fertilization through to the blastula stage. In all figures labelling of the microtubules is with the monoclonal antibody, DM 1A, as the primary antibody and the secondary antibody used was anti-mouse and raised in sheep with FITC conjugated to it. Corresponding staining of the chromosomes (where present) was with  $1 \mu\text{g.ml}^{-1}$  of Hoechst 33258.

Once the oocytes were fertilized, any early changes in microtubule structure and corresponding chromosomal movements are difficult to visualise under the normal fluorescence microscope. This is because the majority of oocytes are visualised in a polar view of the metaphase spindle. This made it difficult to follow any changes as dividing chromosomes were positioned on top of each other. However, the optical sectioning facility of the Confocal Laser Scanning Microscope (CLSM) enabled a series of optical sections (a z-series) of microtubule structures to be taken through the embryo (labelling of the chromosomal material could not be visualised with the confocal system because the laser was not able to excite the Hoechst dyes). This enabled an image of each individual section to be taken at specified distances through the material.

At 10 minutes post-fertilization, Figure 8.1 shows the distribution of microtubules during the formation of the first polar body. Each optical section ( $2.2 \mu\text{m}$  apart) of the z-series has been superimposed onto the previous image, but shifted horizontally by 50 pixels to the right. The images show that the meiotic spindle is inclined into the plane of the image as one spindle pole is in the second image from the

Fig. 8.1. Embryo of *Arenicola marina* at 10 minutes post-fertilization. Composite image of confocal optical sections of 1st polar body formation. Eight optical images were taken  $2.2\ \mu\text{m}$  apart and then each image was superimposed 50 pixels to the right onto the previous image. The polar body is inclined into the plane of the image as one spindle pole is higher than the other. The outline of chromosomes can be seen in the midzone of the polar body in the 4th, 5th and 6th images from the left. Scale bar= $25\ \mu\text{m}$ .



left while the other pole appears in the seventh and eighth image. The spindle itself is 17  $\mu\text{m}$  long from pole to pole and is made up of compact astral microtubules, kinetochore and spindle microtubules. The outline of some of the chromosomes can be seen in the centre of the spindle and seem to be lined up at metaphase. This is confirmed by a micrograph of the corresponding chromosome arrangement (not shown).

In Figure 8.2a (2 hours post-fertilization) microtubules are present in three areas of the embryo. Figure 8.2b shows the distribution of the corresponding chromosomal material. Microtubules have formed into the sperm aster (SA) which surrounds the male pronucleus on the surface of the egg. The male pronucleus is migrating toward what will become the female pronucleus. In the centre of the embryo the 1st polar body (PB1) has already formed, characterised by the condensed ball of chromatin constructed of microtubules (not in focus). Adjacent to the 1st polar body, the 2nd polar body (PB2) is in the process of forming. From Figure 8.2a alone the stage of formation is unclear. However, inspection with the CLSM (image not shown) and the corresponding chromosomes (which are probably in the characteristic “ring” stage) suggest the embryo to be at metaphase of the second meiotic division.

Within approximately 2 hours of fertilization, the formation of both polar bodies is complete, signifying the end of the meiotic divisions. The first mitotic division can be visualised at approximately 3 hours post-fertilization. Figure 8.3a shows the microtubule distribution at the first mitotic division. Astral microtubules are prominent, radiating from each spindle pole and show the unequal cleavage of the embryo. The parallel bundle of microtubules is known as the midzone. The midzone is composed of kinetochore and polar microtubules and is clearly distinguishable, although at this stage, no cleavage furrow can be seen in the embryo. The corresponding chromosome arrangement shows the chromosomes are arranged at metaphase (Fig. 8.3b). To the left of the metaphase chromosomes, and although out of focus, the chromosomal material of the two polar bodies can also be seen. Figures



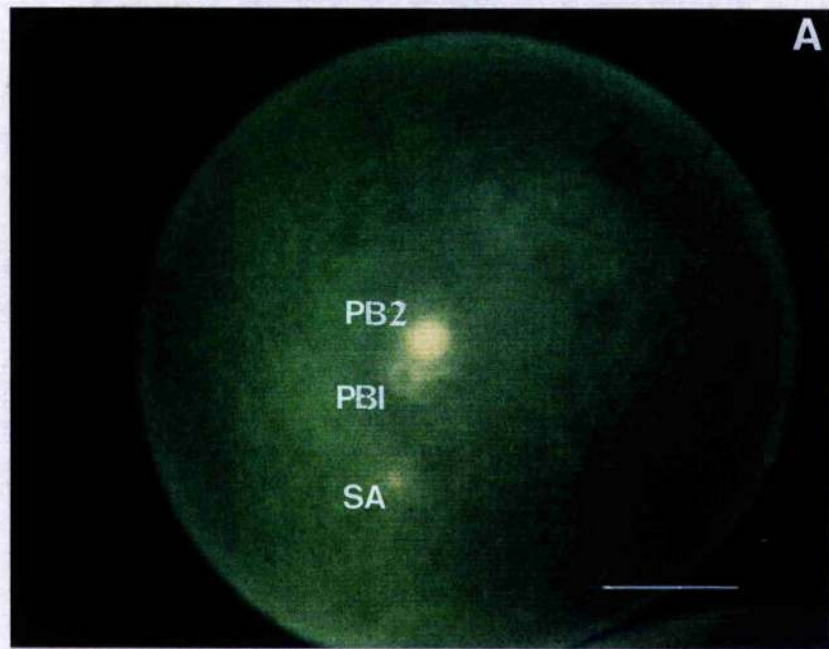


Fig. 8.2. Embryo of *Arenicola marina* at 2 hours post-fertilization. Microtubules are labelled in three distinct areas of the embryo: sperm aster (SA); surrounding the chromosomal material of the 1st polar body (PB1) and metaphase spindle of the formation of the 2nd polar body (PB2) (Fig. 8.2a). Chromosomal material corresponds to the male pronucleus within the sperm aster, 1st polar body and chromosomes on the metaphase plate of the second meiotic spindle; the formation of the 2nd polar body (Fig. 8.2b). Scale bar=50  $\mu$ m.

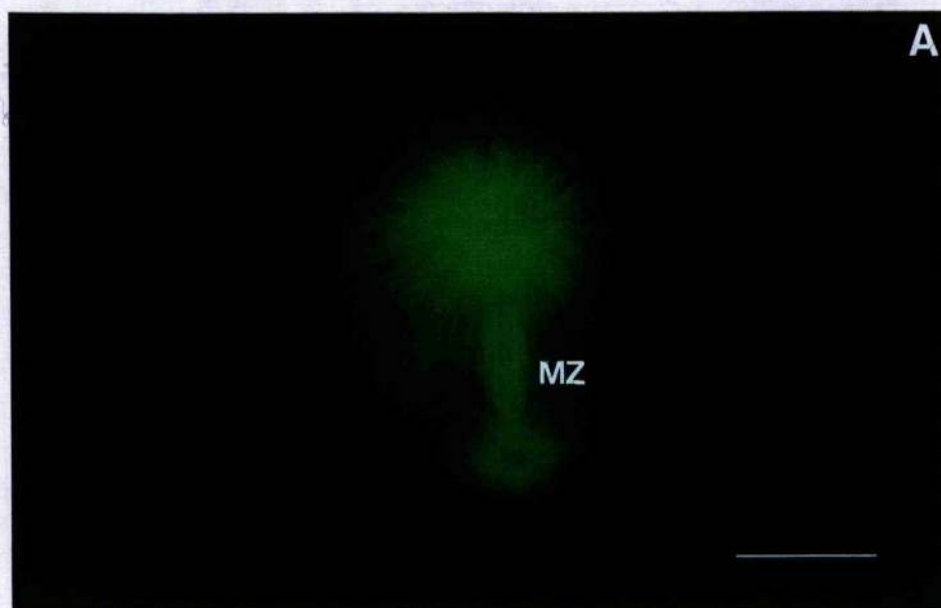


Fig. 8.3. Embryo of *Arenicola marina* at 3 hours post-fertilization. The first mitotic spindle has formed. The midzone (MZ) consists of kinetochore and polar microtubules (Fig. 8.3a). The chromosomes are aligned on metaphase plate and chromosome material of both polar bodies (PBs) is present although out of focus (Fig. 8.3b). Scale bar=50  $\mu$ m.



8.3a and 8.3b are direct prints from the slide film (Ciba Chromes®). These show how much detail and clarity is lost from the original pictures after the normal processing method. However, the expensive nature of these prints prohibits their use throughout this thesis.

Figure 8.4a shows the microtubule arrangement at a later stage (3.5 hours post-fertilization) of the first mitotic division. At this stage, the astral microtubules completely fill each hemisphere of the embryo. The cleavage furrow is visible and bisects the midzone which joins the two cleavage hemispheres just prior to completion of cytokinesis. In the upper hemisphere the second mitotic spindle has just begun to form, perpendicular to the first mitotic spindle. This is indicated by the two brighter areas which correspond to two newly forming spindle poles. In addition, astral spindles have begun to radiate out from these two new poles. The corresponding chromosome arrangement is shown in Figure 8.4b. The chromosomes appearance confirm the embryo to be in the telophase stage of the first mitotic division: The chromosomes have decondensed although the reformed nuclear membrane can not be seen (if present at all). The two polar bodies' chromosomal material is also visible.

The optical sectioning facility of the CLSM enabled a z-series to be taken of an embryo (Fig. 8.5). Each image was taken every 4.4  $\mu\text{m}$  through the embryo which had also reached the telophase of the first mitotic division (4 hours post-fertilization). Image A of Figure 8.5 is the uppermost image of the embryo (nearest the embryo's surface). The polar bodies can be seen as two bright areas on the cleavage furrow. The polar bodies and the cleavage furrow are brightly staining because of the high density of microtubules that were present. The ends of the astral microtubules fill most of each cleavage hemisphere of the embryo.

Image B shows astral microtubules radiating from both hemispheres and the formation of the second mitotic spindle, perpendicular to the first mitotic spindle. This is shown by the two brighter areas corresponding to two new spindle poles and

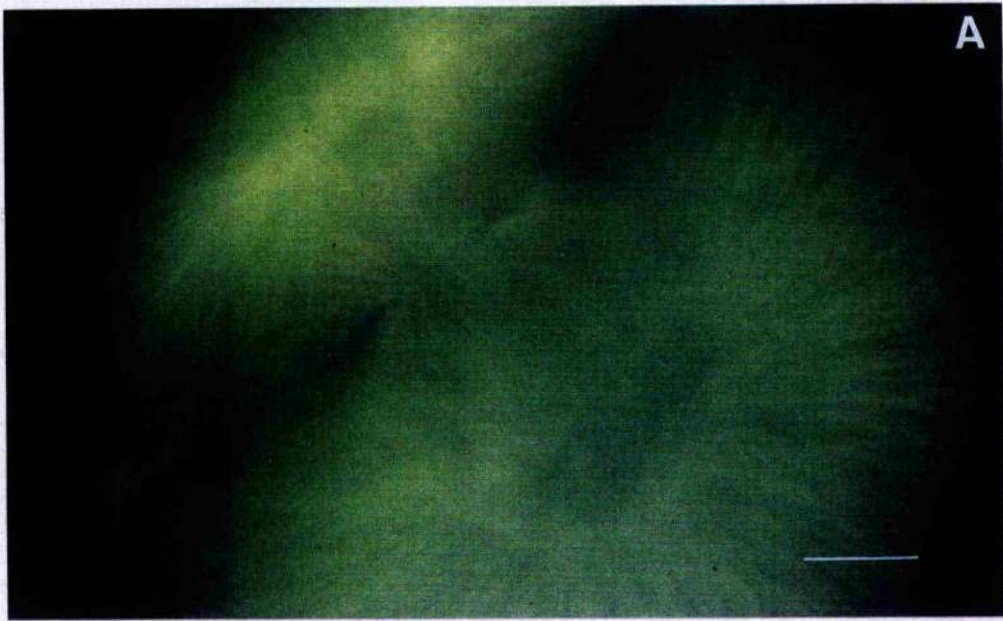
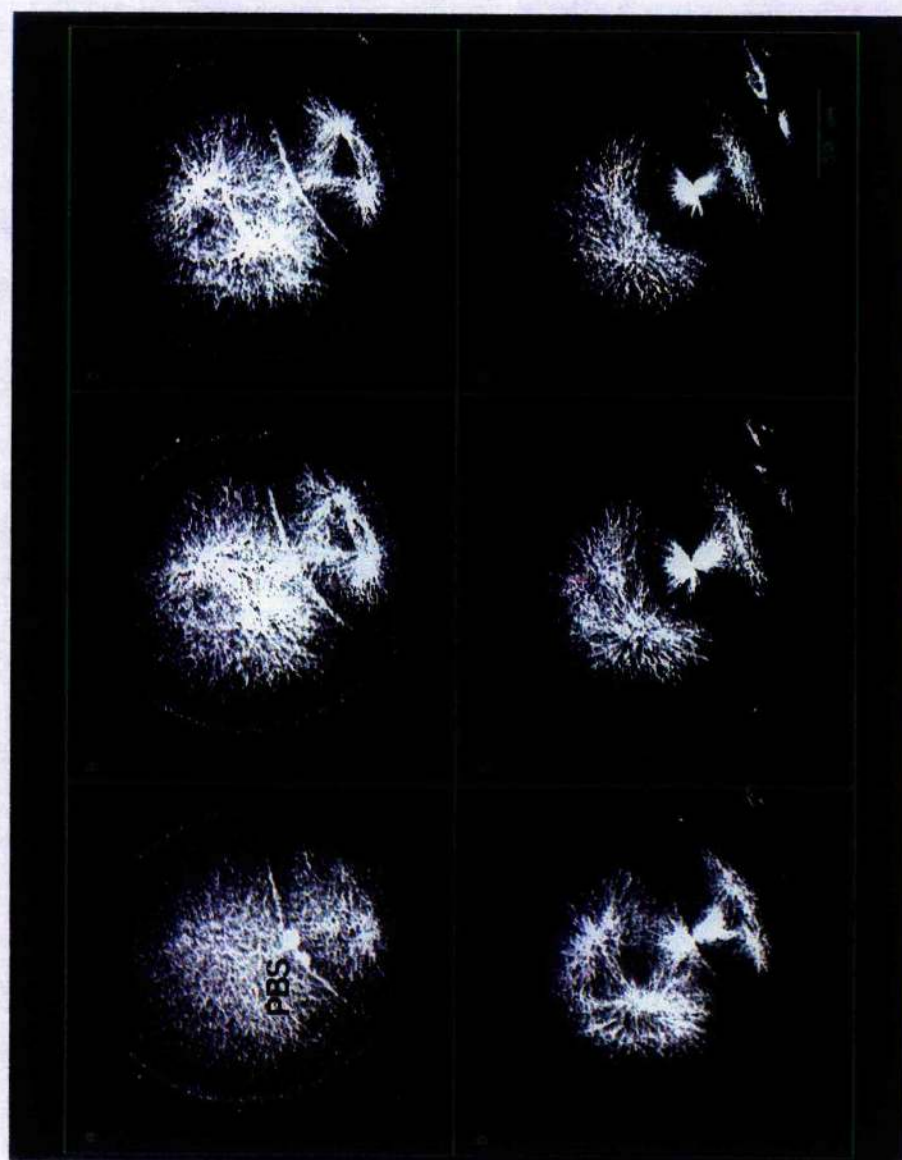


Fig. 8.4. Embryo of *Arenicola marina* at 3.5 hours post-fertilization. The first mitotic spindle has formed with astral microtubules filling each blastomere (Fig. 8.4a). The chromosomes are decondensed and in the telophase configuration (Fig. 8.4b). Scale bar=25  $\mu\text{m}$ .

Fig. 8.5. A confocal micrograph of a z-series of an *Arenicola marina* embryo at 4 hours post-fertilization. The embryo is at telophase of the first mitotic division. Six optical sections (A-F) were taken 4.4  $\mu\text{m}$  apart. Image A is nearest to the oocyte surface (PBS=polar bodies). Scale bar = 50  $\mu\text{m}$ .





astral spindles which have begun to radiate out from these two new poles in the lower hemisphere.

Both new spindle poles in each hemisphere can be distinguished in image C. Polar and kinetochore microtubules of the newly forming second mitotic spindle can also be seen although these cannot be distinguished. In the lower hemisphere the extremities of the midzone can be observed as split between the two newly forming spindle poles.

In image D (13.2  $\mu\text{m}$  further into the embryo from image A) the midzone can be visualised as a dense area of microtubules and has been constricted by the formation of the contractile ring (not labelled). In images E and F the ends of the astral microtubules are in the focal plane of the optical sections and the midbody is clearly constricted in the centre by the contractile ring.

The embryo shown in Figure 8.6a (5 hours post-fertilization) is at the second mitotic division. Astral microtubules fill most of each cleavage hemisphere. Microtubules surrounding the polar bodies can also be seen in the centre of the embryo, although out of focus. The corresponding chromosome arrangement (Fig. 8.6b) shows the embryo to be at the anaphase stage as the sister chromatids have separated and are moving to opposite spindle poles.

At approximately 8 hours post-fertilization most of the embryos have reached the 8 or 16 blastomere stage. In Figure 8.7 each optical section (2.5  $\mu\text{m}$  apart) of the z-series has been overlaid onto the previous image. There are 20 images in total which combined show a reconstruction of all eight spindles within the embryo confirming that the embryo is at the fourth mitotic cycle. Each mitotic spindle can be observed with the two polar bodies in the centre of the embryo. The distribution of the microtubules clearly shows the spiral cleavage and the corresponding micrograph (not shown) of the chromosomes shows all the dividing blastomeres to be at the metaphase stage.

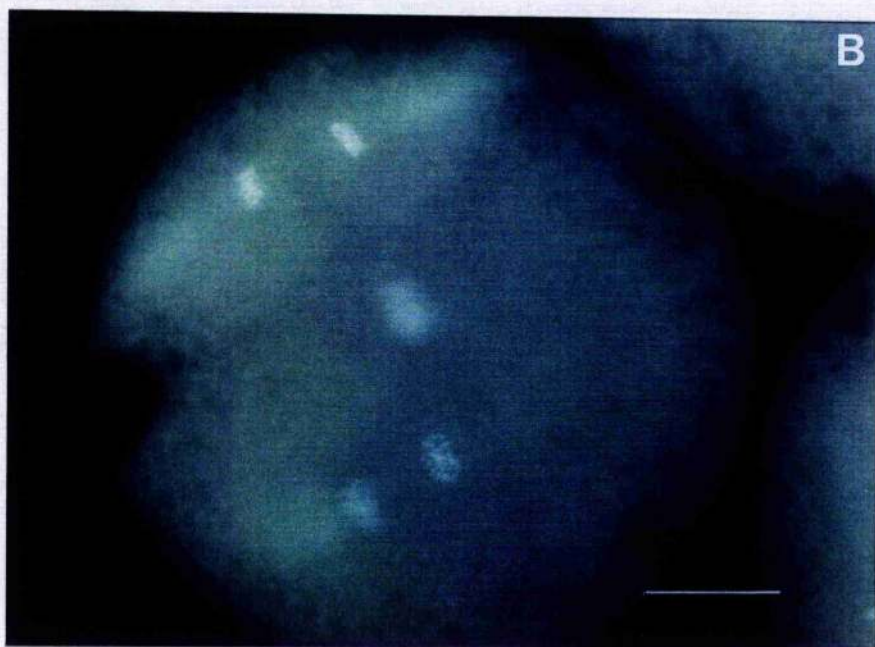
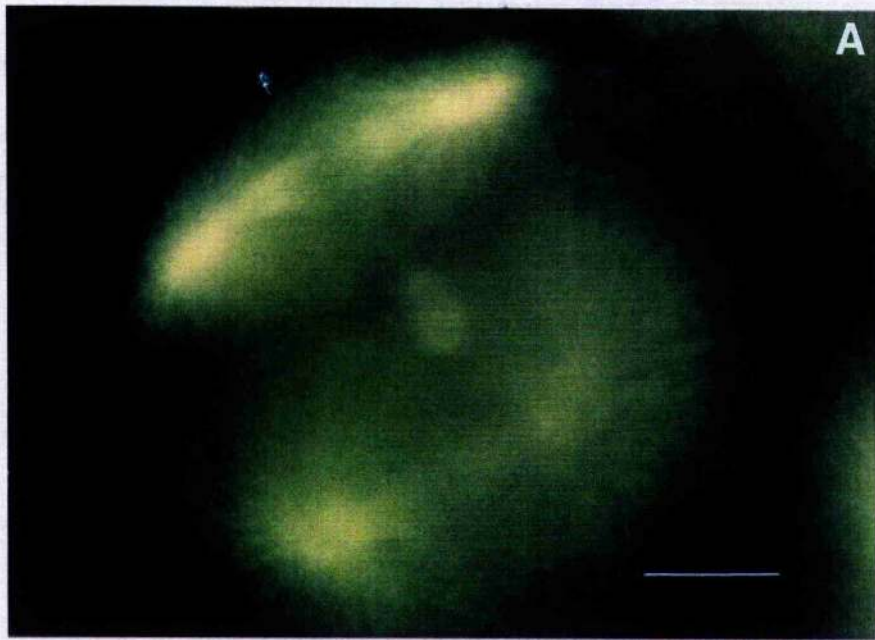
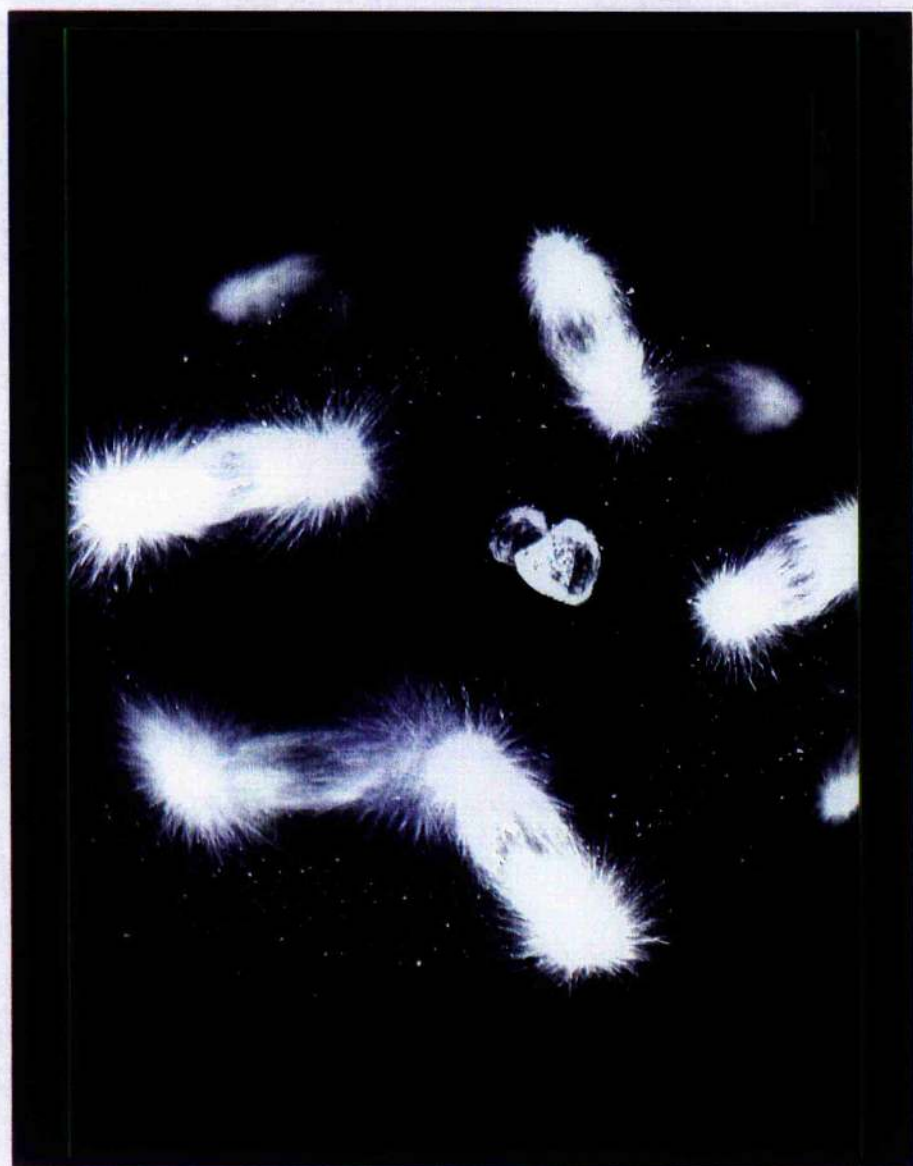


Fig. 8.6. Embryo of *Arenicola marina* at 5 hours post-fertilization. The second mitotic spindle has formed (Fig. 8.6a) and chromosomes are at anaphase, moving to opposite poles (Fig. 8.6b). Scale bar=50  $\mu\text{m}$ .



Fig. 8.7. Embryo of *Arenicola marina* at 8 hours post-fertilization. Composite confocal micrograph of fourth mitotic cycle. Twenty optical images taken  $2.2\ \mu\text{m}$  apart and then each image was superimposed onto the previous image. Polar bodies are visible in the centre of the oocyte and are surrounded by eight mitotic spindles. Each mitotic spindle is at metaphase (image not shown) Scale bar =  $25\ \mu\text{m}$ .



At 24 hours post-fertilization embryos have reached the blastula stage. Even at this late stage microtubule labelling is present. However, it is confined to the edges of the outer blastomeres and mitotic spindles within specific blastomeres (Fig. 8.8a). The mitotic spindles can be seen on the surface of the blastula and these correspond to chromosome arrangements at either metaphase or anaphase (Fig. 8.8b).

From the 50 embryos counted at each fixation time interval, it is possible to look at the kinetics of post-fertilization development and some of the specific stages highlighted above. Figure 8.9 shows the general sequence of changes occurring from fertilization through to blastula in *Arenicola marina* embryos both in terms of chromosomal and microtubule changes.

### *Arenicola defodiens*

The time series of the distribution of microtubules through fertilization and post-fertilization development is incomplete because of the short supply of specimens of *Arenicola defodiens*. An in-detail comparison between *A. marina* and *A. defodiens* was therefore not possible. However, a number of stages were labelled and these are shown in Figs. 8.10 and 8.11.

Figure 8.10a shows the microtubule distribution of an embryo (2 hours post-fertilization) that is at the second meiotic division (formation of the second polar body stage). In the centre of the embryo the 2nd polar body is forming and Figure 8.10b of the corresponding chromosomal distribution shows the embryo to be at the late anaphase stage. The first polar body has already formed characterised by the condensed ball of chromatin surrounded by microtubules. The male pronucleus or corresponding sperm aster cannot be seen in either of these micrographs.

During the second meiotic division an unfamiliar distribution of microtubules was observed in a small number of embryos. A confocal micrograph of this distribution is shown in Figure 8.11. The microtubules are distributed over the surface of the embryo in no recognisable pattern. The corresponding chromosomal



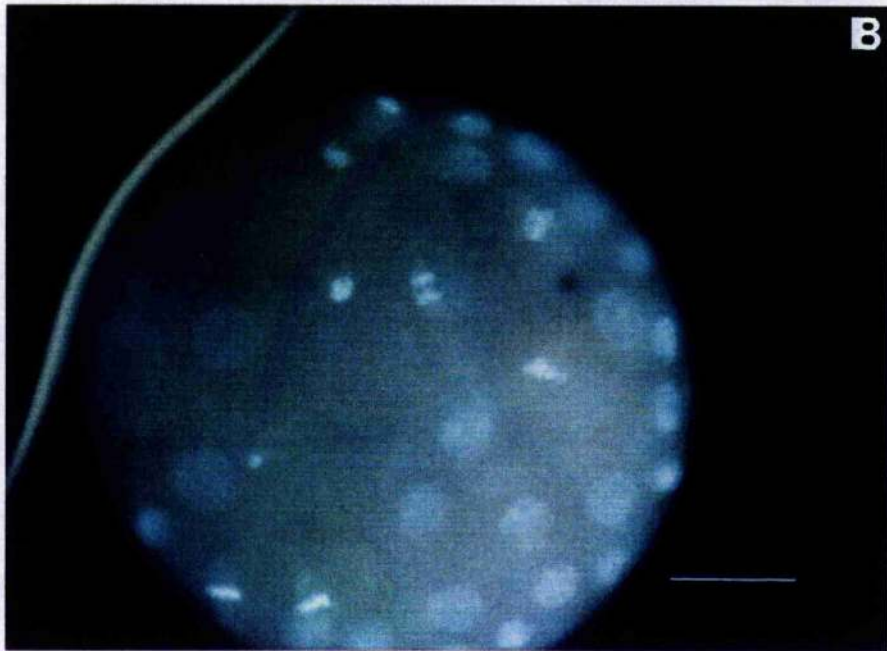
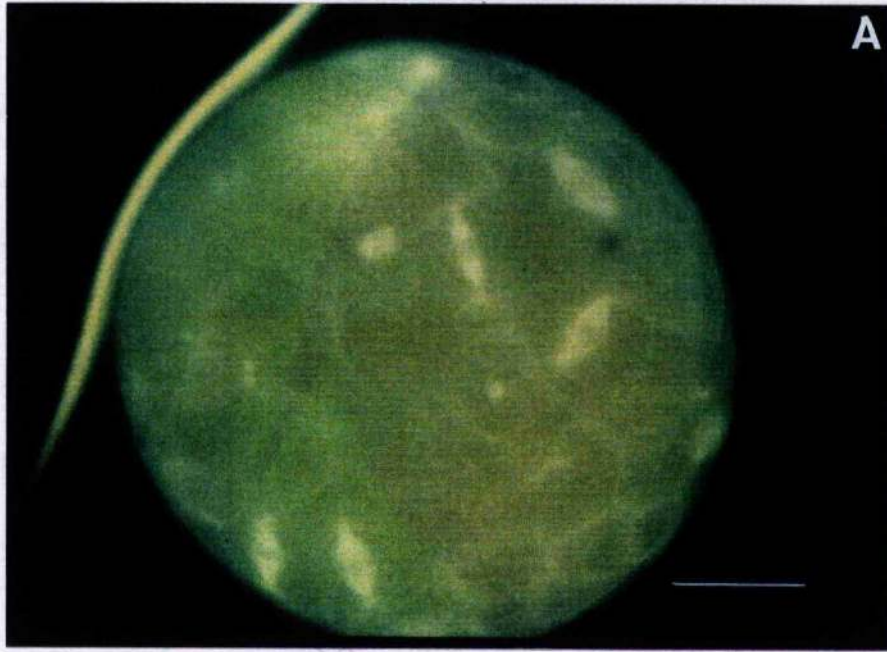


Fig. 8.8. Embryo of *Arenicola marina* at 24 hours post-fertilization. The embryo has reached blastula stage, microtubule labelling is confined to the outer blastomeres. The mitotic spindles are present within individual blastomeres (Fig. 8.8a). The chromosomes are at various stages of mitosis and correspond to mitotic spindles (Fig. 8.8b). Scale bar=50  $\mu$ m.

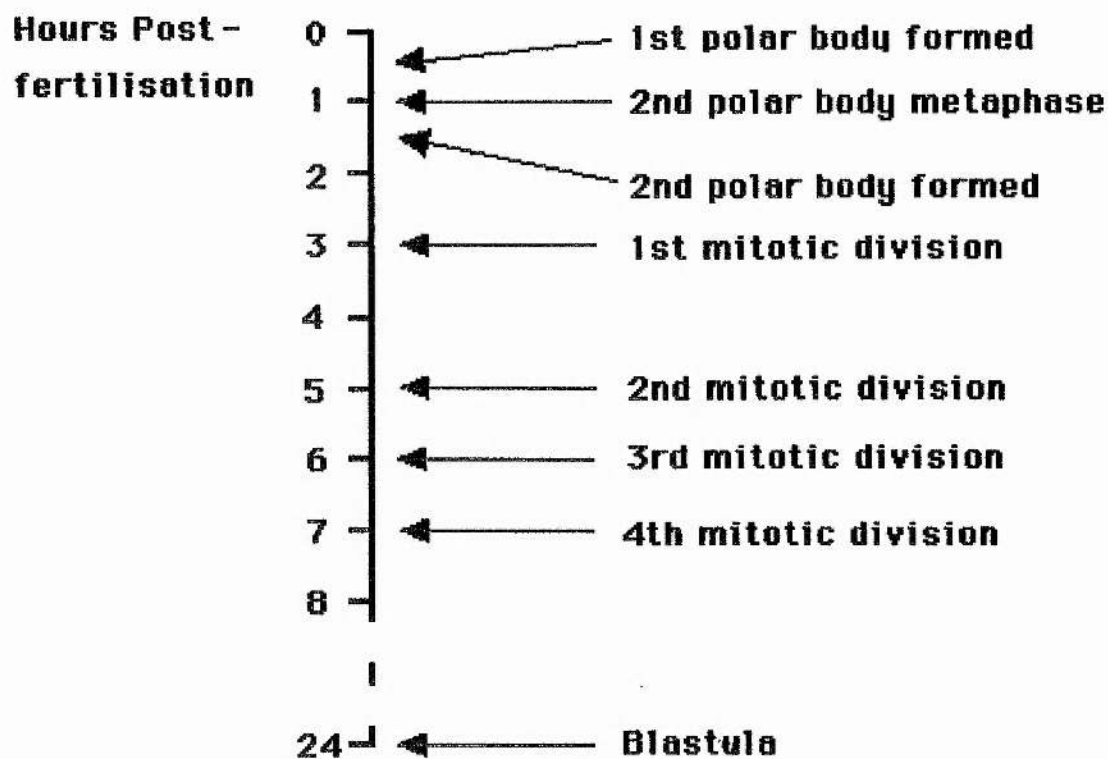


Fig. 8.9. The kinetics of post-fertilization development in relation to microtubule and chromosomal changes in embryos of *Arenicola marina* after *in vitro* maturation with CMF. Times of specific morphological changes are in hours from fertilization and are averaged from 50 embryos.

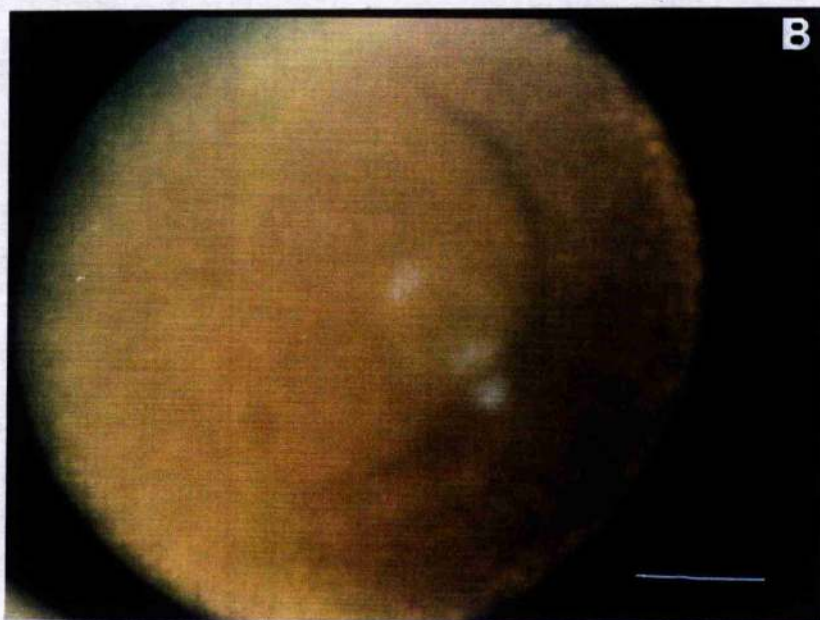
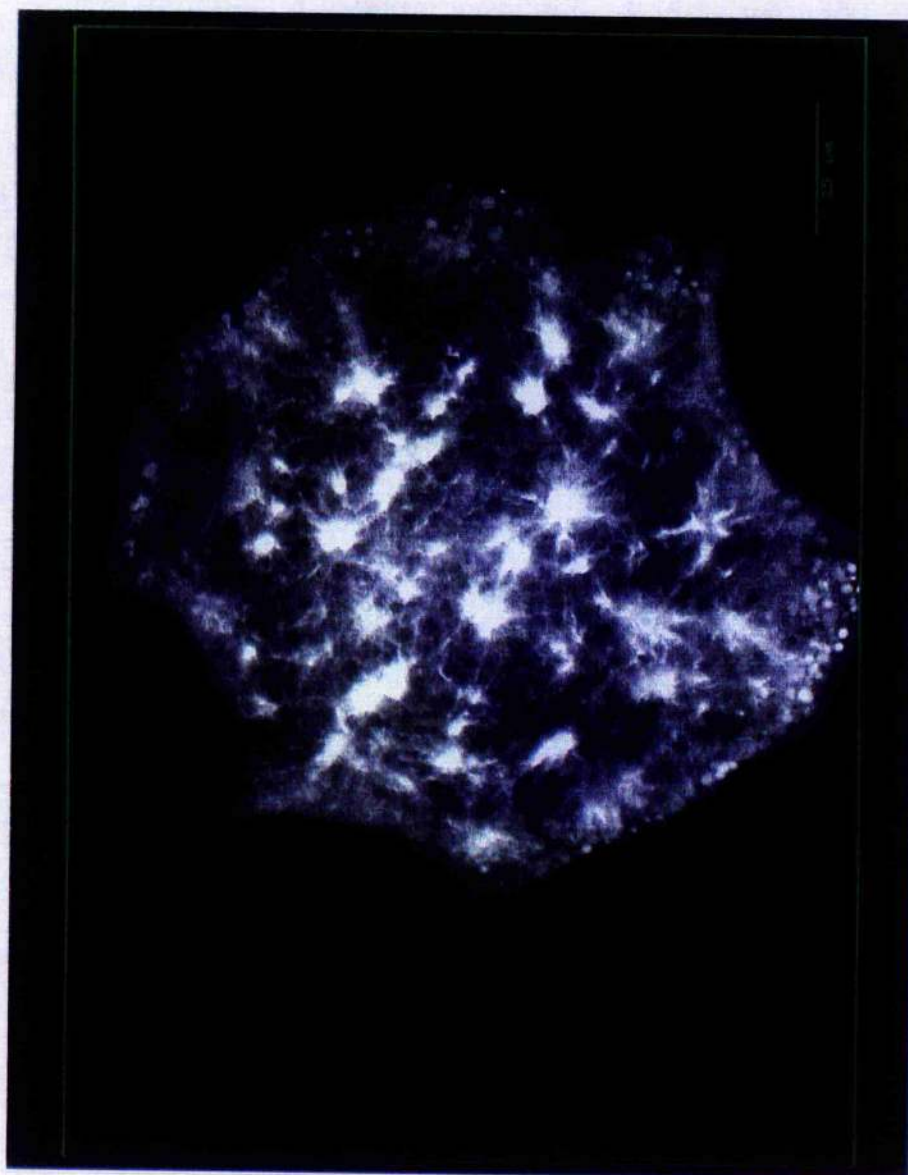


Fig. 8.10. Embryo of *Arenicola defodiens* at 2 hours post-fertilization. The second meiotic spindle has formed (Fig. 8.10a) and chromosomes are moving to opposite poles; in anaphase (Fig.8.10b). Scale bar=50  $\mu$ m.



Fig. 8.11. Embryo of *Arenicola defodiens* at 2 hours post-fertilization. A confocal micrograph of the surface of the embryo at the end of second polar body formation. Microtubules are scattered over surface of oocyte but no corresponding chromosomal arrangement was visualised. Scale bar= 25  $\mu$ m.



distribution shows the embryo to be at the end of the second meiotic division (second polar body formation). However, other chromosomal material corresponding to this specific microtubule distribution is not visible.

### *Nereis virens*

The problems encountered with the fixation and labelling procedure for *Nereis virens* resulted in a time series that was incomplete. Although some microtubule labelling was present in one or two embryos (not shown), the protocol failed to get satisfactory results in the majority of embryos. However, in blastulas that had started swimming, i.e. had lost their jelly coat, fixation and labelling using the technique as described in section 2.2 were excellent and examples are shown in Figure 8.12 and 8.13.

The microtubule distribution of the surface layer of blastomeres of a swimming blastula is shown in Figure 8.12a. The cytoskeletal structure of each blastomere can be seen, although the majority of these blastomeres are at the interphase stage as no mitotic spindles are present. This is confirmed by the decondensed chromosomal material shown by the Hoechst staining in Figure 8.12b. In four of the blastomeres, mitotic spindles are present and these correspond with chromosomes at the metaphase stage as shown in Figure 8.12b. Antibody labelling has also labelled the tubulin of the ciliated band on the outside of the blastula.

Figure 8.13a shows one of the mitotic spindles at a higher magnification. Astral microtubules which radiate out from each pole are distinguishable from the kinetochore and polar microtubules which make up the midzone. However, kinetochore and polar microtubules cannot be distinguished from each other. A gap is observable between the two halves of the midzone and this corresponds to the chromosomes which are aligned in a tight band characteristic of the metaphase stage (Fig. 8.13b).

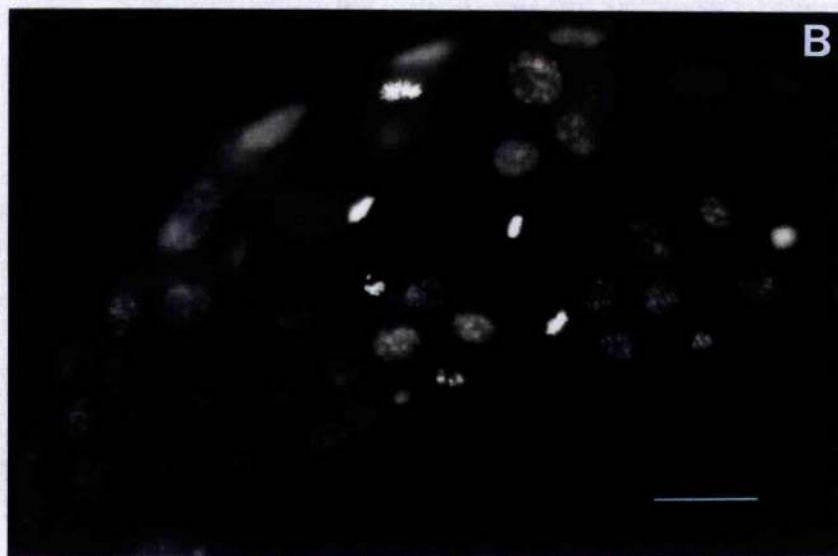
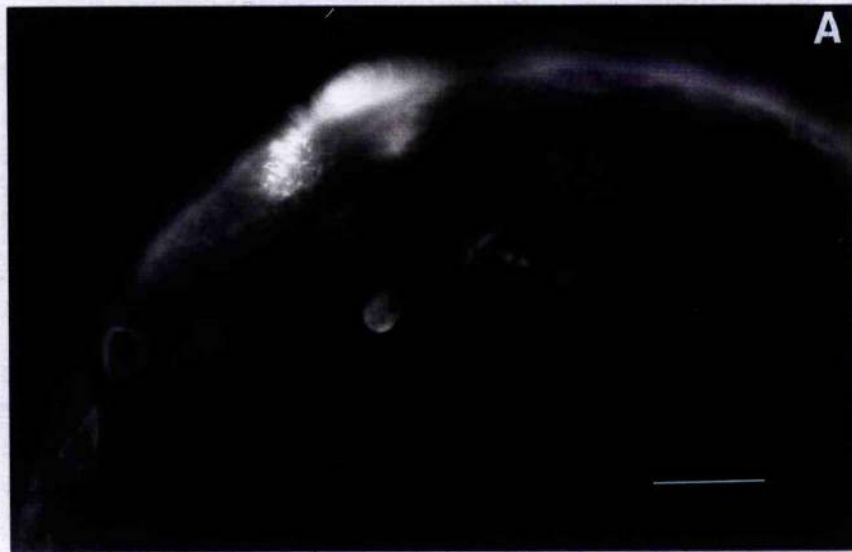


Fig. 8.12. Embryo of *Nereis virens* at 3 days post-fertilization. Embryo has reached swimming blastula stage. The microtubule labelling is confined to the outer blastomeres. Mitotic spindles are present within individual blastomeres. The ciliated band is also labelled (Fig. 8.12a). The chromosomes corresponding to the mitotic spindles are at the metaphase stage of mitosis (Fig. 8.12b). Scale bar=25  $\mu$ m.





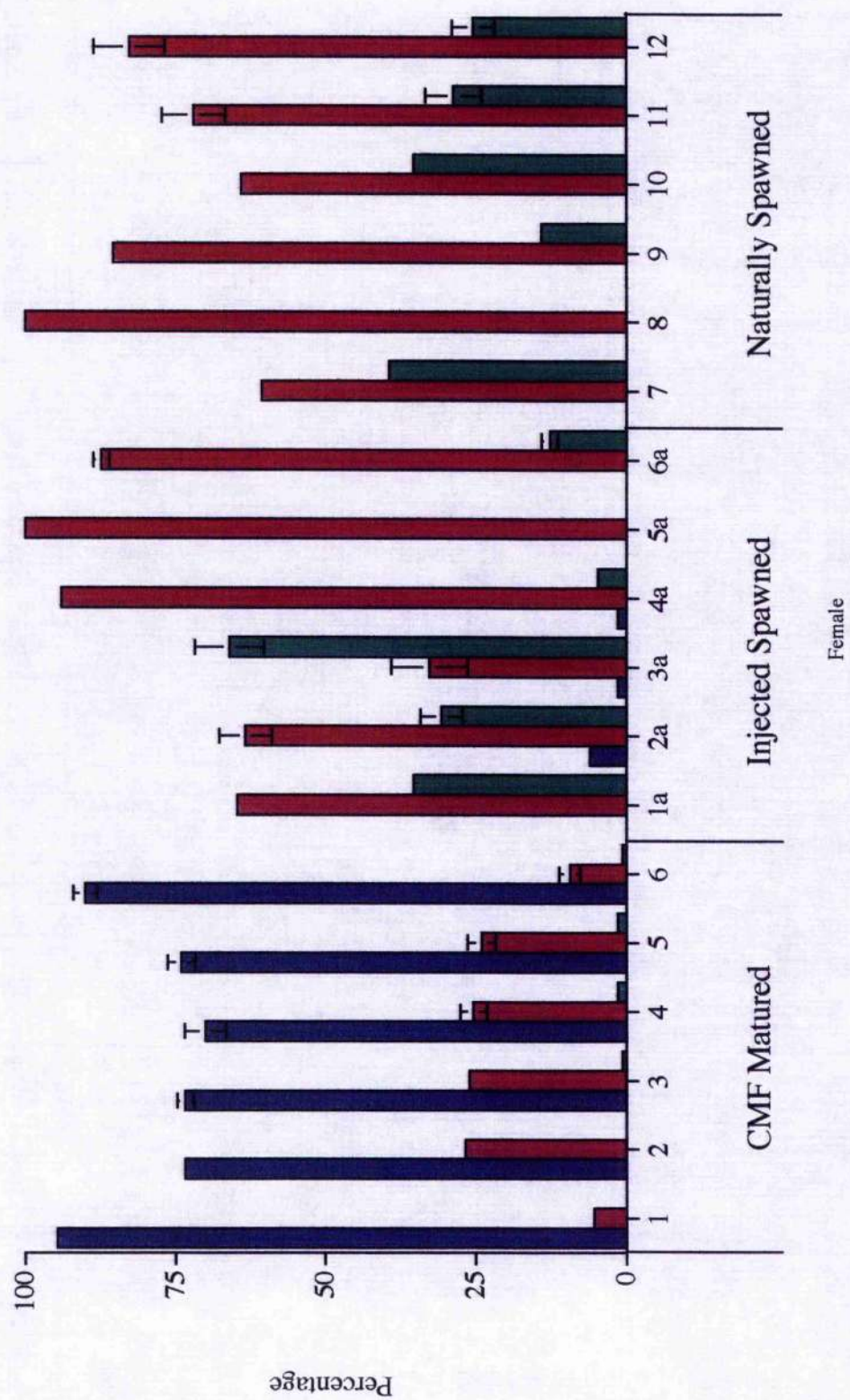
Fig. 8.13. A mitotic spindle of a swimming embryo of *Nereis virens* 3 days post-fertilization. The astral microtubules are clearly distinguishable from the midzone (Fig. 8.13a). The chromosomes are aligned at the metaphase stage of mitosis in the centre of the midzone (Fig. 8.13b). Scale bar=10  $\mu$ m.

### 8.3.2 Effects of Maturation Method on Development Rates in *Arenicola marina*

The results of the effect of different maturation methods on rates of development in *Arenicola marina* (a continuation of the experiment started in section 2.2.4) are shown in Figure 8.14. The complexity of modelling the data presented in Figure 8.14 resulted in confining the analysis to the visual inspection of graphically presented data. Graphical inspection of the results shows that there are wide variations between individual females in the stages of mitosis their embryos have reached at 5 hours post-fertilization. Differences among the three treatments are also clearly present; the *in vitro* maturation of oocytes (Treatment 1) resulted in significantly slower development in all 6 females. Specifically between 73% and 95% of the oocytes matured *in vitro*, from females (1-6), have reached the first mitotic division. Except for a very small proportion (between 0.5% and 1%) the rest of the oocytes are at the second mitotic division. This is approximately one or in some cases two mitotic cycles slower than mature oocytes obtained by injection of prostomial homogenate into a worm which then spawns (Treatment 2) and from naturally spawned females (Treatment 3). Examination of females from treatments 2 and 3 shows that in 5 of the females (1a, 2a, 7, 10, 12) approximately 60% of the embryos have reached the second mitotic division and in four of these females the remaining embryos having reached the third mitotic division (1a, 7, 10, 12). In female 2a, 6% of the embryos are still at the first mitotic division the rest have reached the third division. Embryos of females 5a and 8 are all at the second mitotic division. In females 6a and 9 a small percentage (12%-14%) of embryos have gone onto the third division while the rest remain at the second division. In females 11 and 12, approximately 75% have reached the second mitotic division with the remainder of the embryos at the third. Only in female 3a have more embryos gone onto the third division (66%) than embryos at the second (33%) and first division (1%).



Fig. 8.14. Mean percentage ( $\pm$ S.E.M) of *Arenicola marina* embryos (5 hours post-fertilization) at each mitotic stage (blue bars, first; red bars, second and green bars, third mitotic division) after being matured with different treatments: CMF Matured, oocytes matured after incubation for 3 hours in coelomic fluid with CMF activity; Injected Spawned, oocytes matured after being spawned by females injected with homogenised prostomia solution; Naturally Spawned, oocytes matured after being spawned from naturally spawning females (three counts of 50 embryos per female). Oocytes from females 1-6 were removed for incubation in CMF, prior to injection of homogenised prostomia into those females and subsequent spawning to produce oocytes 1a-6a.



## 8.4 Discussion

### 8.4.1 Sequential Observations of Post-Fertilization Development using Microtubule and Chromosomal Labelling

The continuation and modification of the immunofluorescent labelling technique in Chapter 2 has enabled the microtubule development and chromosomal changes during post-fertilization development in *Arenicola marina*, *A. defodiens* and *Nereis virens* to be examined for the first time.

Normal fluorescence microscopy has provided a time series through fertilization up until the blastula stage of development in *Arenicola marina*. However, at later stages of development the spiral cleavage pattern exhibited in polychaetes means that some resolution is lost because spindles are arranged on top of each other. The use of the confocal laser scanning microscope (CLSM) in parallel with normal fluorescence microscopy overcomes these problems and enables a much more detailed examination of the distribution of microtubules from within the embryo.

The results presented here are only of a selected series of events after fertilization. Although the study of *Arenicola marina* here was comprehensive in that microtubule changes were followed from fertilization through to blastula, certain areas of post-fertilization development were not examined in detail: The formation of sperm aster, male and female pronucleus and their association were not visualised in detail in this study. The labelling of the sperm aster was only seen in some embryos in the cortex region of the embryo. Enlargement of the sperm aster through polar body formation was not visualised, although this may be because the timing of the fixations missed this out.

In *Arenicola marina* the majority of the oocytes with a meiotic spindle were visualised from a polar view. Microtubule structures and chromosomal movements were difficult to visualise because the chromosomes were lying on top of one another.

The CLSM enabled optical sections to be taken overcoming this problem. The formation of both polar bodies was visualised and was complete within approximately 2 hours. Polar bodies can be seen as spherical structures, the framework of which seems to be composed entirely of microtubules. *A. marina* oocytes are fertilized at the metaphase of the first meiotic division, and it is likely (although not proven) that association of the male and female pronuclei follows the *Ascaris* type as described in section 6.1.1. The male and female pronuclei in this type, do not fuse to form the zygote. Instead intermixing after pronuclear membrane disruption occurs on the first mitotic spindle. However, these developments were not recorded during this time series. The first mitotic spindle was the next stage recorded after approximately 3 hours post-fertilization. Astral microtubules can be seen at this stage and trace the unequal cleavage of the blastomeres of an embryo at metaphase. When the different parts of this first mitotic spindle are compared to other marine invertebrates differences can be seen. In the sea urchins, *Lytechinus pictus* and *Strongylocentrotus purpuratus* the astral microtubules at metaphase are much more compact (relative to the size of the midzone) than *A. marina* (CF. Harris *et al.*, 1980; Hollenbeck and Cande, 1985). In the ascidian, *Molgula occidentalis*, the astral spindles are again more compact, but also the mitotic spindle is much shorter than in *A. marina* (CF. Sawada and Schatten, 1988).

As embryos of *Arenicola marina* reach the telophase stage of the first mitotic division (3.5 hours post-fertilization) the astral microtubules fill the whole of each blastomere and the chromosomes have decondensed. This is also the case with *Molgula occidentalis* and *Lytechinus pictus* (Hollenbeck and Cande, 1985; Sawada and Schatten, 1988). In *A. marina* the cleavage furrow can also be seen and CLSM optical sectioning shows that the second mitotic spindle has already begun to differentiate into the two spindle poles (Fig. 8.5). Development continues through spiral cleavages as is typical for protostomes and after 24 hours incubation at 10°C the embryos have reached the blastula stage, although they are not swimming.



Development times in *Arenicola marina* are different from many species of invertebrate and in most cases this is dependent on a whole range of factors; for example oocyte size, inherent differences within the oocytes, temperature range of each species. The comparison of development times of *A. marina* with other species does not therefore warrant discussion.

In *Arenicola defodiens* a similar study of microtubule development was attempted as for *A. marina*. However, the lack of specimens resulted in a limited number of labelled stages and so the post-fertilization development times cannot be compared. In comparison to *A. marina*, the labelling and fixation in *A. defodiens* was reduced in quality. The results showed that in most oocytes and embryos of *A. defodiens*, labelled microtubule structures were less distinct from background labelling and were of a weaker signal than *A. marina*. Hoechst staining also produced weaker signals even though in both cases all protocols were identical for both species. The other major difference was that although fixation protocols were the same, the fixed oocytes and embryos from *A. defodiens* were often not spherical and fixation was of a lower quality, for example Figure 8.11. An explanation for these differences may be because of inherent differences between the two species in the chemical and structural composition of the oocytes. Another possible explanation is that with the limited number of specimens used for fertilization of *A. defodiens*, the specimens may have been inferior and not representative of oocytes from this species in general. The lack of well labelled embryos prevented the direct comparison of post-fertilization development between *A. defodiens* and *A. marina*. However, the visualisation of an unknown distribution of microtubules between the end of the second meiotic division and before the first mitotic division was not visualised in any of the *A. marina* embryos examined. Explanations for this anomaly could be that the microtubules visualised are the beginnings of the first mitotic spindle. This is not likely, however, because the spindle would grow from the spindle poles where the microtubule organising centres (MOTCs) were present and not from the periphery inwards.

In the case of labelling embryos of *Nereis virens*, the jelly coat was already known to interfere with the fixation of embryos in electron microscopy (Bass and Brafield, 1972). To achieve fixation and labelling of post-fertilization stages, a number of different experimental approaches were attempted. However, even the most successful fixation and labelling technique, as described in section 8.2.5, failed to achieve consistent labelling. It is likely that this technique was also inadequate in producing good labelling because of its failure to remove the jelly coat completely. This supposition is based on the fact that labelling of swimming blastulas, that had hatched and therefore lost their jelly coat, was excellent. Surface microtubules and mitotic spindles within the surface blastomere cell layers as well as the ciliated band were all labelled.

#### **8.4.2 Effects of Maturation Method on Development Rates in *Arenicola marina***

Different maturation methods have already been shown to have no significant effect on oocyte maturation specifically the diameter of the first meiotic spindle and corresponding diameter of the chromosome ring (section 2.3.3). It was therefore surprising to find that *in vitro* maturation of oocytes had such a profound effect on the rate of post-fertilization development. When oocytes were matured *in vitro* with CMF, the stage of development was found to be one or sometimes two mitotic cycles behind oocytes from naturally spawning females and oocytes matured by injecting females with prostomial homogenate which were then allowed to spawn. Nearly all the embryos from both these treatments had reached either the second or third mitotic division as opposed to *in vitro* matured embryos, the majority of which were still at the first mitotic division (Fig. 8.14).

These differences could be ascribed to inter-individual variation between oocytes from different females. Clearly, variation in the numbers of embryos at each mitotic stage is present between females within each treatment. However, clear overall differences exist between the different treatments which may also be due to



inter-individual variation. Differences between embryos from naturally spawning females and *in vitro* matured embryos could be explained by inter-individual variation, although for two groups of 6 females to all show the same differences through natural variation between females is extremely unlikely. However, the evidence that confirms that inter-individual variation cannot be responsible for the observed differences in developmental rates is that oocytes matured *in vitro* were removed from the same females that were subsequently injected with prostomium.

Although every effort was made to fertilize oocytes from different treatments at the same time, the unpredictability of spawning of females in the experiment resulted in oocytes from females 2a, 3a, 8 and 9 being 1 hour older when fertilized than the other females' oocytes. Therefore, the age of oocytes may also have been a possible reason for the observed differences. However, oocytes from only these females or from the other females both reflect the overall pattern that *in vitro* matured oocytes are slower than the other two treatments. The age at which oocytes are fertilized is therefore also not responsible for the observed differences between treatments.

It is therefore apparent that the differences in the speed of post-fertilization development is due to the method used to mature the oocytes. The reduction in the rate of post-fertilization development of *in vitro* matured oocytes may be the result of a number of different factors. Firstly, the speed of each mitotic cycle may be reduced. Maturing oocytes *in vitro* may act by reducing the speed of the whole cycle or may act on only one stage. Another possible area that could be influenced by *in vitro* maturation is the actual incorporation of the sperm and subsequent association of the male and female pronucleus. In this case only the rate of these particular events are reduced so inducing a lag effect which is maintained through mitosis. A lag effect could also be introduced if the sperm required more time to find and penetrate the oocyte.

No other directly comparable work has been performed in other species either invertebrate or vertebrate species. Related work in the Surf Clam *Spisula solidissima*,

by Kuriyama *et al.* (1986), examined the differences in the speed of development between fertilized oocytes and oocytes parthenogenetically activated with KCl. These authors found that although GVBD and first meiotic spindle formation occurred at the same rate, the formation of polar bodies was quicker in parthenogenetically activated oocytes. In rabbits and mice the timing of oocyte maturation was also examined (Chang, 1955; Thibault, 1972). In these vertebrates maturation can be induced *in vitro* and *in vivo*. Although these comparisons are problematic because of the unknown time of induction of maturation in *in vivo* studies, these authors showed that differences between the timing of *in vivo* maturation of follicle enclosed oocytes and *in vitro* maturation in isolation are minimal.

From the results presented in section 8.3.2 and the lack of comparable work, biochemical or molecular explanations as to why maturing oocytes *in vitro* would slow the mitotic cycle or components of the cycle are difficult to formulate. This is also the case with male pronucleus formation and association with female pronucleus. However, the possibility that sperm may have more difficulty in finding *in vitro* matured oocytes could be explained if the oocytes matured *in vitro* were less "attractive" to the sperm. The common factor in naturally spawned oocytes and oocytes spawned after injection of prostomial homogenate is that in both treatments oocytes pass through the nephromixia. The nephromixia may activate or add a factor on the oocytes' surface which makes them more "attractive" to sperm. *In vitro* matured oocytes which have not passed through the nephromixia would therefore be less "attractive". Sperm would therefore spend more time "looking" for oocytes and so fertilization would be delayed. No proof exists that oocytes undergo any changes when passing through the nephromixia. However, it is well known that chemotaxis of sperm to oocytes occurs in a number of phyla. In Echinoderms a peptide called resact, isolated from the egg jelly layer of *Arbacia punctulata*, has chemotactic properties for sperm as have compounds isolated from oocytes of the coral *Montipora digitata* (Ward *et al.*, 1985; Coll *et al.*, 1990). To date no compounds have been isolated from oocytes of annelids, but the possibility remains that a chemoattractive

molecule is activated or added to the surface of the oocyte as it passes through the nephromixia.

## **Chapter 9**

### **General Discussion**

## 9.1 Introduction

The objectives of this thesis, as stated in Section 1.9, cover a number of areas, each of which may be regarded as a discrete topic. The various Chapters, each address particular aspects of oocyte maturation and its control, fertilization and post-fertilization development. Together these form a chronological study of early development in two species of polychaete; *Arenicola marina* and *Nereis virens*.

## 9.2 The Control of Oocyte Maturation in *Arenicola marina*

The use of the fluorescent Hoechst dyes has enabled an *in vitro* assay to be developed which can be used to assess the levels of maturation inducing activity in coelomic fluid of *Arenicola marina* and other substances. Auckland (1993) suggested that a second substance was required to induce maturation in oocytes of *A. marina*. Experimental results, presented in this thesis, demonstrate that a substance found within the coelomic fluid is the second step required after the prostomium maturation substance for oocyte of *A. marina* to mature. The evidence for this two-step process is given as follows: Firstly, the *in vitro* incubation of oocytes directly in prostomial homogenate fails to induce maturation. However, the *in vitro* incubation of oocytes in coelomic fluid from a female, that has previously been injected with homogenised prostomia, induces maturation. The possibility that the injected prostomial homogenate is acting on the oocytes directly, whilst within the animal, can also be discounted from results presented in section 4.2: These results show that in the majority of females injected with prostomial homogenate, maturation inducing activity is not present within the coelomic fluid immediately after injection of the prostomial homogenate. The prostomial maturation substance may instead of inducing the production of a second substance, be auto-amplified within the

coelomic fluid to a concentration that induces oocyte maturation directly. However, it seems unlikely that a substance produced in one part of the body (the prostomium) would then act on another separate target to produce more of the same substance.

### **9.3 Different Systems of Oocyte Maturation in *Arenicola marina* and *A. defodiens***

Results presented in this thesis may seem to contradict earlier work by Meijer and Durchon (1977) and Meijer (1979a,b 1980). However, a number of lines of evidence suggest that these authors were probably working on the closely related species recently described as *Arenicola defodiens* (Cadman and Nelson-Smith, (1993). Oocyte maturation in specimens from Northern France could be induced by incubation directly in prostomial homogenate (Meijer and Durchon, 1977). In this thesis, British populations have been shown to require two steps to induce maturation. Meijer (1980) also found a number of chemicals that induced maturation in specimens collected from Northern France. Results in this thesis have shown that oocytes of British populations could not be induced to mature with the same chemicals and concentrations as Meijer's specimens.

These differences in oocyte maturation suggest different systems of maturation and it has been confirmed that rather than contradicting earlier work, worms collected from Meijer's sampling sites have been identified as *Arenicola defodiens* (Bentley, pers. comm.) Further proof that Meijer's sampling population was composed of *A. defodiens* is also found in his thesis, as he noted that oocytes from populations other than his sampling site, sometimes failed to mature when incubated directly in prostomial homogenate maturation (Meijer, 1978).



## 9.4 Coelomic Maturation Factor (CMF): Similarities to other Maturation Inducers

The maturation inducing activity shown to be present in the coelomic fluid of *Arenicola marina* has been termed the Coelomic Maturation Factor (CMF). A number of experiments carried out during the course of this study have begun to characterise its production, mode of action and chemical structure, and these characteristics can be compared and contrasted with other systems.

CMF, like 1-methyl adenine (1-MeAde) in starfish is produced by the action of a different substance from another source. In starfish, Gonad Stimulating Substance (GSS) acts on the follicle cells surrounding the oocytes which then produce 1-MeAde *de novo* (Kanatani, 1969; Kanatani *et al.*, 1969; Kanatani and Shirai, 1970; Kanatani, 1972; Kanatani and Shirai, 1972; Schuetz 1972; Shirai and Kanatani, 1972; Hirai *et al.*, 1973). The experiments to examine whether CMF is also produced *de novo* after the injection of prostomial homogenate or is already present as a precursor in the coelomic fluid prior to spawning, however, failed to produce conclusive results.

In starfish, the follicle cells surrounding the oocytes produce 1-MeAde (Hirai *et al.*, 1973). Although CMF is found within the coelomic fluid, the exact site of production is unknown. Oocytes in *Arenicola marina* develop freely within the coelomic fluid without follicle cells. The site of CMF production may therefore be either another tissue or cells within the coelomic fluid. Coelomocytes of other species have already been shown to be involved in vitellogenin production; e.g. *Nereis virens* (Fischer and Rabien, 1986; Baert and Slomianny, 1987). In *A. marina* coelomocytes have a phagocytic role (Clark and Clark, 1962). It is feasible therefore to suggest that coelomocytes may also be involved in CMF production.

The mode of action of both CMF and 1-MeAde are in some ways also similar. The minimum incubation time required for both these substances to induce

maturation is approximately 20 minutes and is temperature dependent in starfish. The incubation of CMF at higher temperatures significantly reduces its activity, but its affect on the incubation time required to induce maturation is unknown. As the kinetics of both CMF and 1-MeAde are similar, it might be assumed that the pathways, by which they act, may also be the same. In starfish, a number of pathways, especially those involving changes in intracellular free calcium, have been implicated. In starfish and many other species, calcium has been shown to be involved in maturation, possibly acting as a second messenger to activate Maturation Promoting Factor (MPF). Santella and Kyozuka (1994) are only the latest of a number of authors to suggest that in starfish, an increase in calcium is essential for maturation to proceed. Other workers, however, have suggested that changes in calcium are present in starfish at maturation, but are not necessary or sufficient to induce maturation (Kikuyama and Hiramoto; 1991 Stricker *et al.*, 1994). In this study, ionophore A23187 as well as a number of other calcium agonists fail to induce maturation in *Arenicola marina*. This suggests, as some work on starfish has, that a calcium increase is not involved, or at least is not sufficient to induce maturation in *A. marina*. A similar story exists for disulphide reducing agents where these have been reported to induce maturation in *A. defodiens* and starfish (Kanatani and Kishimoto, 1973; Meijer, 1980), but fail to induce maturation in *A. marina* as shown in this study.

Experiments on the chemical structure of CMF have indicated that it is likely to be a protein. When maturation inducers are compared across phyla, a wide variety of molecular types exist, but only in *Sabellaria alveolata* and *Pectinaria gouldii* have proteins been implicated as maturation inducers. In *S. alveolata* three types of digestive enzymes from the neutral serine class have been identified (Peaucellier, 1977). In *P. gouldii* a 12 kDa, thermolabile substance from the cement gland suggests a substance of proetinaceous nature. It may be that in polychaetes as with serotonin in molluscs and 1-MeAde in starfish that proteins as maturation inducers are a common theme across this phyla.

The production, mode of action and chemical structure of CMF have been shown to have a number of similarities with maturation systems of other phyla. At present, however, the results from this thesis fail to show any clear and direct link between the properties of CMF and a specific system in another phylum suggesting that a new system may be present in *Arenicola marina*.

## 9.5 Microtubule Changes During Maturation and Post-Fertilization Development

Immunocytochemical techniques have been used for the first time in *Arenicola marina* to label microtubules through maturation and post-fertilization. This has generated a time series of observations through maturation and post-fertilization enabling meiotic and mitotic structures to be followed and to be characterised. Parallel investigations in *A. defodiens* and *Nereis virens* were incomplete and so comparisons between the three species were not possible.

The time series through maturation and fertilization in *Arenicola marina* has also enabled the effects of different methods of maturation on microtubule structures and the speed of development to be investigated. The *in vitro* maturation of oocytes of *Arenicola marina* with CMF, results in a slower rate of development of oocytes compared with oocytes matured *in vivo*; i.e. spawned naturally or after the injection of homogenised prostomia into a female. No similar studies have been carried out in other phyla and reasons for the observed differences therefore remain speculative. No differences were observed in the diameters of the meiotic spindle after different maturation levels.

## 9.6 Calcium at Fertilization

The investigation of the signals controlling maturation and fertilization within oocytes of *Arenicola marina* and *Nereis virens*, specifically, changes in the internal calcium concentration were investigated using a microinjection system. Due to circumstances beyond control, only preliminary investigations were possible, mainly involving the verification of the microinjection system. Early indications suggest that although fertilization and development within the microinjection chamber are normal, the injection procedure has, in some cases, serious effects on fertilization and subsequent development. Early experiments indicating changes in intracellular free calcium were, with further inspection, due to artefacts of the microscopical examination and not related to fertilization. The establishment of the technique during this study should provide a sound basis for further experimentation.

## 9.7 *Arenicola marina* and *Nereis virens*; Model Systems for Maturation and Post-Fertilization Development?

In other phyla such as Echinodermata and Mollusca many of the species, lend themselves to the study of oocyte maturation. A number of qualities have contributed to their extensive utilisation including; the abundance of individuals, the ease of obtaining large amounts of synchronised gametes and an extended season in which mature adults can be obtained. The ease that which maturation in an oocyte can be visualised, however, is the most significant reason to use these species. In echinoderms and some molluscs GVBD, for example, can be easily visualised with normal light microscopy because of the transparency of the oocytes. In amphibians, the presence of an easily visualised white spot signifies that maturation is complete. Consequently these phyla have become established model systems for maturation,

with the result that work on many of these phyla has reached the molecular level of control with identification of maturation inducers and specific pathways and components they act upon already resolved. For example the maturation inducer, 1-methyladenine (1-MeAd) in starfish, was identified over 25 years ago (Kanatani, 1969; Kanatani *et al.*, 1969; Kanatani and Shirai, 1970; Hirai *et al.*, 1973).

In recent years with new developments, especially the increased use of DNA labelling dyes such as the Hoechst series, workers have begun to move away from these "easy" species. A wide variety of species' oocytes have now been used in maturation studies. Previously these species were not utilised for the same reason as *Arenicola marina* oocytes; that is maturation could not be observed clearly. This study has for the first time used the DNA labelling dyes in *Arenicola marina* to provide a reliable and quick method of scoring maturation. With the development of the assay, a reliable method for scoring maturation has now been fulfilled as the final criteria for a good model system. *A. marina* already meets the other criteria for a model system because a) a number of populations of *Arenicola marina* around British coasts spawn at different times providing mature adults for up to 3-4 months a year; b) the abundance of specimens in a population and c) the easy collection of adults as well as investigations into the culturing of *A. marina* will also increase the supply of mature individuals. In protostomes, *A. marina* now fulfils all the criteria to become a model system for maturation. It may be argued that another model system is not necessary for protostomes as a number of molluscs already fulfil this role. The maturation processes described in this thesis, however, show that the method of maturation is very different from molluscs and so the use *A. marina* as a model system in parallel to the other already established systems would be justified. Within the polychaetes, *Chaetopterus* sp. have also been investigated in detail and have been proposed as a model system (Eckberg and Anderson, 1995). However, at this moment in time oocyte maturation in this species is induced by an unknown trace component in sea water. Clearly, the partial characterisation of CMF makes *A. marina* a more attractive model system because the elucidation of CMF's structure

could be used to look at transduction of signals, its production and action on receptors etc. all of which would be impossible in *Chaetopterus* sp.

The immunocytochemical study of maturation and post-fertilization development in *Arenicola marina* has shown that for changes in microtubule structures and its relation to development, *A. marina* could also be used as a model system. As with the control of oocyte maturation, the majority of work on microtubules has been on echinoderms and molluscs. The development of *A. marina* as a model system for microtubule changes could also run in parallel to these already established systems.

In *Nereis virens* a contrasting model system of maturation is present: this system is not under endocrine control and maturation is induced by fertilization. As *N. virens* is cultured commercially, mature animals are available for a number of months. In addition each animal contains an abundance of gametes making this species an ideal model system for protostomes, but in Category 1. The systems investigated could be pathways involved in fertilization, specifically the role of calcium. The use of a model system for microtubules is, however, impossible until consistent and good labelling of microtubules can be obtained in stages where the jelly coat is still present.

## 9.8 Areas for Future Research

The results presented in this thesis have provided a sound basis for continued investigations into maturation, fertilization and post-fertilization development in both *Arenicola marina* and *Nereis virens*. This research has proposed a number of new and interesting questions requiring investigation:

- i) Continued identification and characterisation of CMF in *Arenicola marina* using other purification and separation techniques including High Performance Liquid



Chromatography (HPLC) and a repeat of SDS-Polyacrylamide Gel Electrophoresis to elucidate the structure of CMF.

ii) The examination of the mode of action of CMF, specifically a number of areas could be investigated. The separation of coelomic fluid into component parts and the incubation of prostomial homogenate with the different parts of the coelomic fluid *in vitro* should pinpoint the site of production. An investigation into the receptor sites and pathways CMF acts on using microinjection of fluorescent calcium probes could look at the role of calcium in maturation. The further investigation into the kinetics of CMF induced maturation; including effects of temperature on the incubation time of oocytes in CMF required to induce maturation.

iii) A continuation of the investigation into the role of calcium at fertilization in both *Nereis virens* and *Arenicola marina*. This would primarily involve the refinement of the microinjection technique to eliminate effects of the injection procedure on fertilization and development. Confocal imaging of oocytes injected with fluorescent dyes and the use of calcium agonists would then be used to investigate the role of calcium.

iv) Investigations have already shown that *Arenicola marina* oocytes, up to 8 days old after spawning, can be fertilized and develop to blastula (much longer than any other marine invertebrate broadcast spawner) (M. E. Williams pers. comm.). In vertebrates a protein kinase, termed cytostatic factor (CSF), maintains the arrest at meiosis II by associating with  $\beta$  tubulin (Bray, 1992). What controls the metaphase arrest in *Arenicola marina* and what mechanisms are involved in maintaining a competent meiotic spindle for 8 days? Is an inability to fertilize after 8 days due to the failure to maintain the meiotic spindle so inducing subsequent breakdown? An investigation into these areas would begin to answer other fundamental questions about the control of oocyte maturation.

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# Appendix 1

## Solutions

### **Phosphate Buffered Solution (PBS) Azide 10x stock solution made up in DW (Store at Room Temp)**

NaCl	128 mM
KCl	20 mM
Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O	80 mM
KH <sub>2</sub> PO <sub>4</sub>	20 mM
Sodium Azide	10 mM

### **PBS Buffer (PBS Azide+0.1% Triton X-100 (Store at 4°C)**

PBS azide 10x stock solution	1 ml
Triton X-100 10% solution in DW	0.1 ml
Distilled water	8.9 ml

#### *Purpose of Components*

1. PBS- suitable buffer for antibody work
2. Azide-prevents bacterial contamination
3. Triton X-100 permeabilises membranes to allow entry of antibodies

### **Jelly Coat Removal Solution (Store at Room Temp)**

NaCl	500 mM
KCl	10 mM
NaHCO <sub>3</sub>	2 mM
EGTA	25 mM
pH adjusted to 8.5 with Na <sub>2</sub> CO <sub>3</sub>	

### **Mounting Fluid (Store at -20°C)**

Glycerol	9 ml
Tris base 1 M solution	20 µL
p-Phenylenediamine	0.1% w/v
(dissolve the p-Phenylenediamine in the Tris base first)	

### *Purpose of Components*

1. Tris base- Buffer
2. p-Phenylenediamine- anti fading agent, facilitates storage of slides

### **Dents Fixative (Store at -20°C)**

Methanol	80%
Dimethyl sulphoxide (DMSO)	20%
Add EGTA	50 mM

### *Purpose of Components*

1. Methanol- fixative and allows entry of antibodies
2. DMSO- as for methanol
3. EGTA- designed to reduce calcium in the solution to approximately 1 mM, because of sensitivity of microtubules to the presence of  $\text{Ca}^{2+}$ , which may cause disassembly.

### **Formaldehyde Fix (Store constituent parts separately at 4°C)**

Formaldehyde	2%
PIPES pH 6.8	20 mM
EGTA	5 mM
$\text{MgSO}_4$	0.5 mM
Triton X-100	0.1%

### *Purpose of Components*

1. Formaldehyde- fixation of sample
2. EGTA- designed to reduce calcium in the solution to approximately 1 mM, because sensitivity of microtubules to the presence of  $\text{Ca}^{2+}$ , which may cause disassembly.
3. PIPES- a favourable buffer for retaining microtubule structures.
4. Triton X-100- permeabilises cell membrane facilitating entry of antibodies to bind to protein.

### **Non-Fixing PIPES Buffer (Store constituent parts separately at 4°C)**

PIPES pH 6.8	20 mM
EGTA	5 mM
$\text{MgSO}_4$	0.5 mM
Triton X-100	0.1%

### **Dilution Medium**

Sheep serum diluted to 1% in PBS Azide TX100 (0.1%) buffer

The sheep serum prevents most non specific binding of the antibody to the Eppendorf tube used for storage by binding these sites itself.

### **Artificial Seawater**

Made up according to Baloun and Morse (1984)

NaCl	423 mM
CaCl <sub>2</sub>	9.25 mM
MgCl <sub>2</sub>	22.94 mM
MgSO <sub>4</sub>	25.5 mM
NaHCO <sub>3</sub>	2.15 mM
KCl	9.0 mM

### **Calcium Free Seawater**

Made up according to Kuriyama *et al.* (1986)

NaCl	462 mM
MgCl <sub>2</sub> . 6H <sub>2</sub> O	19.7 mM
MgSO <sub>4</sub> . 7H <sub>2</sub> O	45 mM
NaHCO <sub>3</sub>	4.8 mM
KCl	9.0 mM

pH adjusted to 8.0 with 1 M Na<sub>2</sub>CO<sub>3</sub>

### **Trypsin Solution**

Trypsin	50 mg
---------	-------

Type: II-S Porcine Pancreas

(1000-2000 BAEE units.mg.solid)

TFSW	0.5 ml
------	--------

Vortex before use

### **Trypsin Inhibitor Solution**

Trypsin Inhibitor	50 mg
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Type II-S: Soybean

(1 mg will inhibit 1.6 mg of trypsin with 10000 BAEE units)

TFSW	0.5 ml
------	--------

Vortex before use

### **SDS (Sodium-Dodecyl-Sulphate) Buffer (Store at 4°C)**

Dissolves protein samples to give a final concentration of 2 mg.ml<sup>-1</sup> for SDS Gel Electrophoresis.

Tris-HCl pH 6.75	60mM
SDS	2%
Glycerol	10%
b-mercaptoethanol	1%
Bromphenol Blue	0.001%

#### *Purpose of Components*

1. Tris-HCl- buffer
2. SDS- denatures proteins
3. b-mercaptoethanol- disassociates proteins
4. Glycerol- makes solution denser than water to facilitate injection into gel
5. Bromphenol blue- stains solution blue for easy visualisation

### **Intracellular Buffer for Microinjection**

KCl	0.5 M
PIPES	20 mM
EGTA	100 $\mu$ M
pH adjusted to 6.8 with 1 M HCl	

#### *Purpose of Components*

KCl- maintains intracellular ionic concentration  
EGTA- reduces calcium in the solution  
PIPES- a favourable buffer

## Appendix 2

### Tables of Means and Raw Data for Figures 4.9, 4.10, 5.1, 5.2, 5.3, 5.4 and 5.5.

Data for Figure 4.9. Incubation time required for CMF to induce oocyte maturation

Treatment	Mean Percent Matured	Arcsine Mean Percent Matured	s.e. of Arcsine Mean Percent	Arcsine Mean Percent Matured +/- s.e.m	Back Transformed Values	Back Transformed Mean Percent Matured +/- s.e
1 5 min	0.05	1.311	0.909	2.22 1.311 0.402	0.0015 0.0005 0	+0.1 0.05 0
2 10 min	40.46	39.248	3.674	42.922 39.248 35.574	0.4634 0.4046 0.3389	+5.88 40.46 -6.57
3 20 min	78.96	62.685	4.693	67.648 62.685 58.262	0.8560 0.7896 0.7239	+6.64 78.96 -6.57
4 30 min	75.30	60.216	4.654	64.87 60.216 55.562	0.8201 0.7530 0.6808	+6.71 75.30 -7.22
5 40 min	77.38	61.596	2.484	64.008 61.596 59.112	0.8078 0.7738 0.7363	+3.4 77.38 -3.75
6 50 min	80.37	63.684	4.622	68.306 63.684 59.062	0.8633 0.8037 0.7363	+5.96 80.37 -6.74
7 60 min	84.86	67.083	4.279	71.362 67.083 62.804	0.8983 0.8486 0.7911	+4.97 84.86 -5.95
8 80 min	80.09	63.475	3.836	67.311 63.475 59.559	0.8486 0.8009 0.7439	+4.77 80.09 -5.70
9 100 min	76.79	61.221	4.173	65.394 61.221 57.048	0.8280 0.7679 0.7050	+6.01 76.79 -6.29
10 180 min	90.35	71.821	1.785	73.656 71.871 70.086	0.9212 0.9035 0.8841	+1.77 90.35 -1.94
11 TFSW	0	0	0	0	0	0

	treatment	female	% count	Input Column
1	1	1	0.00	
2	1	1	.04	
3	1	1	0.00	
4	1	2	0.00	
5	1	2	0.00	
6	1	2	0.00	
7	1	3	0.00	
8	1	3	0.00	
9	1	3	0.00	
10	1	4	0.00	
11	1	4	0.00	
12	1	4	0.00	
13	1	5	0.00	
14	1	5	.02	
15	1	5	0.00	
16	2	1	.48	
17	2	1	.52	
18	2	1	.44	
19	2	2	.46	
20	2	2	.42	
21	2	2	.42	
22	2	3	.08	
23	2	3	.06	
24	2	3	.04	
25	2	4	.40	
26	2	4	.46	
27	2	4	.50	
28	2	5	.76	
29	2	5	.64	
30	2	5	.60	
31	3	1	1.00	
32	3	1	1.00	
33	3	1	1.00	
34	3	2	.56	
35	3	2	.60	
36	3	2	.60	
37	3	3	.40	
38	3	3	.42	
39	3	3	.44	
40	3	4	.74	
41	3	4	.78	
42	3	4	.72	
43	3	5	.86	
44	3	5	.94	
45	3	5	.94	
46	4	1	.90	
47	4	1	.98	
48	4	1	.92	
49	4	2	.48	
50	4	2	.54	
51	4	2	.52	
52	4	3	.28	
53	4	3	.32	
54	4	3	.32	
55	4	4	.94	
56	4	4	.88	
57	4	4	.90	
58	4	5	.90	



	treatment	female	% count	Input Column
59	4	5	.94	
60	4	5	.92	
61	5	1	.90	
62	5	1	.92	
63	5	1	.88	
64	5	2	.82	
65	5	2	.80	
66	5	2	.76	
67	5	3	.54	
68	5	3	.56	
69	5	3	.60	
70	5	4	.70	
71	5	4	.64	
72	5	4	.60	
73	5	5	.90	
74	5	5	.88	
75	5	5	.90	
76	6	1	.90	
77	6	1	.92	
78	6	1	.94	
79	6	2	.88	
80	6	2	.90	
81	6	2	.90	
82	6	3	.28	
83	6	3	.20	
84	6	3	.24	
85	6	4	.92	
86	6	4	.90	
87	6	4	.92	
88	6	5	.88	
89	6	5	.90	
90	6	5	.92	
91	7	1	1.00	
92	7	1	.96	
93	7	1	.94	
94	7	2	.90	
95	7	2	.96	
96	7	2	.84	
97	7	3	.40	
98	7	3	.44	
99	7	3	.30	
100	7	4	.84	
101	7	4	.88	
102	7	4	.80	
103	7	5	.98	
104	7	5	.96	
105	7	5	.90	
106	8	1	.92	
107	8	1	.90	
108	8	1	.92	
109	8	2	.64	
110	8	2	.56	
111	8	2	.60	
112	8	3	.46	
113	8	3	.54	
114	8	3	.40	
115	8	4	.86	
116	8	4	.86	

	treatment	female	% count	Input Column
117	8	4	.92	
118	8	5	.96	
119	8	5	.98	
120	8	5	.98	
121	9	1	.60	
122	9	1	.66	
123	9	1	.60	
124	9	2	.92	
125	9	2	.90	
126	9	2	.80	
127	9	3	1.00	
128	9	3	1.00	
129	9	3	.98	
130	9	4	.64	
131	9	4	.60	
132	9	4	.60	
133	9	5	.60	
134	9	5	.44	
135	9	5	.50	
136	10	1	.92	
137	10	1	.92	
138	10	1	.94	
139	10	2	.78	
140	10	2	.78	
141	10	2	.76	
142	10	3	.98	
143	10	3	.98	
144	10	3	.96	
145	10	4	.88	
146	10	4	.86	
147	10	4	.86	
148	10	5	.92	
149	10	5	.88	
150	10	5	.96	
151	11	1	0	
152	11	1	0	
153	11	1	0	
154	11	2	0	
155	11	2	0	
156	11	2	0	
157	11	3	0	
158	11	3	0	
159	11	3	0	
160	11	4	0	
161	11	4	0	
162	11	4	0	
163	11	5	0	
164	11	5	0	
165	11	5	0	

Data for Figure 4.10. The Effect of dilution on CMF Activity

Treatment	Mean Percent Matured	Arcsine Mean Percent Matured	s.e. of Arcsine Mean Percent	Arcsine Mean Percent Matured +/- s.e.m	Back Transformed Values	Back Transformed Mean Percent Matured +/- s.e
1 100%	47.03	43.274	3.084	46.358 43.274 40.19	0.5244 0.4703 0.4166	+5.41 47.03 -5.37
2 80%	36.22	37.037	2.790	39.827 37.037 34.247	0.4097 0.3622 0.3176	+4.75 36.22 -4.46
3 60%	29.98	33.180	2.621	35.801 33.180 30.559	0.3422 0.2998 0.2591	+4.24 29.98 -4.07
4 40%	16.93	24.263	2.949	27.212 24.263 21.314	0.2089 0.1693 0.1320	+3.96 16.93 -3.73
5 20%	10.28	18.722	2.310	21.032 18.722 16.412	0.1284 0.1028 0.0797	+2.56 10.28 -2.31
6 10%	1.66	7.414	1.664	9.078 7.414 5.75	0.0250 0.0166 0.0102	+0.84 1.66 -0.64
7 5%	0.64	4.664	1.330	5.994 4.664 3.334	0.0109 0.0064 0.0033	+0.45 0.64 -0.31
8 1%	0.05	1.261	0.879	2.14 1.261 0.382	0.0013 0.0005 0	+0.08 0.05 -0.05
9 TFSW	0.22	2.725	1.488	4.213 2.715 1.237	0.0054 0.0022 0.0004	+0.32 0.22 -0.18

	treatment	female	% count	Input Column
1	1	1	.26	
2	1	1	.30	
3	1	1	.32	
4	1	2	.46	
5	1	2	.40	
6	1	2	.36	
7	1	3	.28	
8	1	3	.26	
9	1	3	.30	
10	1	4	.84	
11	1	4	.80	
12	1	4	.88	
13	1	5	.36	
14	1	5	.36	
15	1	5	.30	
16	1	6	.60	
17	1	6	.66	
18	1	6	.66	
19	2	1	.18	
20	2	1	.20	
21	2	1	.20	
22	2	2	.60	
23	2	2	.60	
24	2	2	.56	
25	2	3	.18	
26	2	3	.20	
27	2	3	.14	
28	2	4	.66	
29	2	4	.60	
30	2	4	.66	
31	2	5	.26	
32	2	5	.20	
33	2	5	.24	
34	2	6	.46	
35	2	6	.36	
36	2	6	.40	
37	3	1	.08	
38	3	1	.08	
39	3	1	.14	
40	3	2	.28	
41	3	2	.20	
42	3	2	.28	
43	3	3	.14	
44	3	3	.14	
45	3	3	.12	
46	3	4	.50	
47	3	4	.50	
48	3	4	.56	
49	3	5	.48	
50	3	5	.46	
51	3	5	.40	
52	3	6	.44	
53	3	6	.40	
54	3	6	.46	
55	4	1	.12	
56	4	1	.14	
57	4	1	.12	
58	4	2	.24	



	treatment	female	% count	Input Column
59	4	2	.24	
60	4	2	.24	
61	4	3	0.00	
62	4	3	0.00	
63	4	3	.04	
64	4	4	.40	
65	4	4	.42	
66	4	4	.40	
67	4	5	.10	
68	4	5	.08	
69	4	5	.08	
70	4	6	.36	
71	4	6	.32	
72	4	6	.30	
73	5	1	.04	
74	5	1	.04	
75	5	1	.02	
76	5	2	.34	
77	5	2	.32	
78	5	2	.26	
79	5	3	.02	
80	5	3	.02	
81	5	3	0	
82	5	4	.18	
83	5	4	.20	
84	5	4	.16	
85	5	5	.08	
86	5	5	.08	
87	5	5	.14	
88	5	6	.14	
89	5	6	.12	
90	5	6	.08	
91	6	1	0	
92	6	1	0	
93	6	1	.02	
94	6	2	.08	
95	6	2	.04	
96	6	2	.08	
97	6	3	0	
98	6	3	0	
99	6	3	0	
100	6	4	.02	
101	6	4	.02	
102	6	4	0	
103	6	5	.02	
104	6	5	.02	
105	6	5	0	
106	6	6	.10	
107	6	6	.10	
108	6	6	.04	
109	7	1	0	
110	7	1	0	
111	7	1	0	
112	7	2	.04	
113	7	2	.04	
114	7	2	.02	
115	7	3	.02	
116	7	3	.02	

	treatment	female	% count	Input Column
117	7	3	0.00	
118	7	4	0.00	
119	7	4	0.00	
120	7	4	0.00	
121	7	5	0.00	
122	7	5	0.00	
123	7	5	0.00	
124	7	6	.06	
125	7	6	.06	
126	7	6	.02	
127	8	1	0.00	
128	8	1	0.00	
129	8	1	0.00	
130	8	2	0.00	
131	8	2	0.00	
132	8	2	0.00	
133	8	3	0.00	
134	8	3	0.00	
135	8	3	0.00	
136	8	4	0	
137	8	4	0	
138	8	4	0	
139	8	5	0	
140	8	5	0	
141	8	5	0	
142	8	6	.04	
143	8	6	.04	
144	8	6	0	
145	9	1	0	
146	9	1	0	
147	9	1	0	
148	9	2	0	
149	9	2	0	
150	9	2	0	
151	9	3	0	
152	9	3	0	
153	9	3	0	
154	9	4	0	
155	9	4	0	
156	9	4	0	
157	9	5	0	
158	9	5	0	
159	9	5	0	
160	9	6	.08	
161	9	6	.10	
162	9	6	.06	



Data for Figure 5.1. The Effects of Incubation for 1 Hour at Different Temperatures on the Activity of CMF

Treatment	Mean Percent Matured	Arcsine Mean Percent Matured	s.e. of Arcsine Mean Percent	Arcsine Mean Percent Matured +/- s.e.m	Back Transformed Values	Back Transformed Mean Percent Matured +/- s.e
1 Immed	54.71	47.714	2.770	50.484 47.714 44.944	0.5954 0.5471 0.4983	+4.83 54.71 -4.88
2 Ice	45.47	42.439	2.373	44.813 42.439 40.066	0.4965 0.4547 0.4149	+4.18 45.47 -3.98
3 5°C	47.21	43.357	3.803	47.160 43.357 40.454	0.5384 0.4721 0.4218	+6.63 47.21 -5.03
4 10°C	49.48	44.708	3.166	47.874 44.708 41.542	0.5505 0.4948 0.4391	+5.57 46.48 -5.57
5 RT	29.50	32.867	3.213	36.080 32.867 29.654	0.3472 0.2950 0.2455	+5.22 29.50 -4.95
6 37°C	11.36	19.863	2.633	22.266 19.663 17.030	0.1440 0.1136 0.0855	+3.04 11.36 -2.81
7 TFSW	0	0	0	0	0	0

	treatment	female	% count	Input Column
1	1	1	.34	
2	1	1	.34	
3	1	1	.30	
4	1	2	.60	
5	1	2	.40	
6	1	2	.70	
7	1	3	.20	
8	1	3	.20	
9	1	3	.20	
10	1	4	.64	
11	1	4	.66	
12	1	4	.60	
13	1	5	.60	
14	1	5	.60	
15	1	5	.54	
16	1	6	.40	
17	1	6	.44	
18	1	6	.50	
19	2	1	.26	
20	2	1	.24	
21	2	1	.28	
22	2	2	.58	
23	2	2	.56	
24	2	2	.56	
25	2	3	.10	
26	2	3	.08	
27	2	3	.10	
28	2	4	.90	
29	2	4	.84	
30	2	4	.80	
31	2	5	.54	
32	2	5	.54	
33	2	5	.58	
34	2	6	.58	
35	2	6	.48	
36	2	6	.58	
37	4	1	.44	
38	4	1	.44	
39	4	1	.46	
40	4	2	.48	
41	4	2	.48	
42	4	2	.56	
43	4	3	.18	
44	4	3	.20	
45	4	3	.24	
46	4	4	.92	
47	4	4	.88	
48	4	4	.86	
49	4	5	.48	
50	4	5	.48	
51	4	5	.40	
52	4	6	.46	
53	4	6	.40	
54	4	6	.44	
55	5	1	.18	
56	5	1	.20	
57	5	1	.12	
58	5	2	.32	

	treatment	female	% count	Input Column
59	5	2	.30	
60	5	2	.26	
61	5	3	.08	
62	5	3	.04	
63	5	3	.06	
64	5	4	.56	
65	5	4	.52	
66	5	4	.48	
67	5	5	.22	
68	5	5	.20	
69	5	5	.22	
70	5	6	.66	
71	5	6	.64	
72	5	6	.60	
73	6	1	.04	
74	6	1	.06	
75	6	1	.08	
76	6	2	.06	
77	6	2	.08	
78	6	2	.08	
79	6	3	.02	
80	6	3	.04	
81	6	3	.08	
82	6	4	.36	
83	6	4	.34	
84	6	4	.32	
85	6	5	.02	
86	6	5	.02	
87	6	5	.02	
88	6	6	.32	
89	6	6	.30	
90	6	6	.30	
91	7	1	0	
92	7	1	0	
93	7	1	0	
94	7	2	0	
95	7	2	0	
96	7	2	0	
97	7	3	0	
98	7	3	0	
99	7	3	0	
100	7	4	0	
101	7	4	0	
102	7	4	0	
103	7	5	0	
104	7	5	0	
105	7	5	0	
106	7	6	0	
107	7	6	0	
108	7	6	0	

Data for Figure 5.2. The Effect of heating on the Activity of CMF

Treatment	Mean Percent Matured	Arcsine Mean Percent Matured	s.e. of Arcsine Mean Percent	Arcsine Mean Percent Matured +/- s.e.m	Back Transformed Values	Back Transformed Mean Percent Matured +/- s.e
1 CMF	46.16	42.797	1.123	43.97 42.797 41.674	48.08 46.16 44.08	+1.92 46.16 -2.03
2 CMF Heated	0	0	0	0	0	0
3 TFSW	0	0	0	0	0	0

	Treatment	Female	% Count	Input Column
1	1	1	.46	
2	1	1	.48	
3	1	1	.58	
4	1	2	.58	
5	1	2	.56	
6	1	2	.56	
7	1	3	.48	
8	1	3	.46	
9	1	3	.48	
10	1	4	.40	
11	1	4	.40	
12	1	4	.44	
13	1	5	.46	
14	1	5	.46	
15	1	5	.52	
16	1	6	.28	
17	1	6	.36	
18	1	6	.36	
19	2	1	0.00	
20	2	1	0	
21	2	1	0	
22	2	2	0	
23	2	2	0	
24	2	2	0	
25	2	3	0	
26	2	3	0	
27	2	3	0	
28	2	4	0	
29	2	4	0	
30	2	4	0	
31	2	5	0	
32	2	5	0	
33	2	5	0	
34	2	6	0	
35	2	6	0	
36	2	6	0	
37	3	1	0	
38	3	1	0	
39	3	1	0	
40	3	2	0	
41	3	2	0	
42	3	2	0	
43	3	3	0	
44	3	3	0	
45	3	3	0	
46	3	4	0	
47	3	4	0	
48	3	4	0	
49	3	5	0	
50	3	5	0	
51	3	5	0	
52	3	6	0	
53	3	6	0	
54	3	6	0	



Data for Figure 5.3. The Effect of Evaporation and Subsequent Rehydration of Coelomic Fluid on CMF Activity

Treatment	Mean Percent Matured	Arcsine Mean Percent Matured	s.e. of Arcsine Mean Percent	Arcsine Mean Percent Matured +/- s.e.m	Back Transformed Values	Back Transformed Mean Percent Matured +/- s.e
1 CF	81.87	64.817	4.584	69.401 64.817 60.233	0.8762 0.8187 0.7530	+5.25 81.87 -6.57
2 Evap CF	3.7	11.132	2.212	13.436 11.132 9.102	0.0537 0.0371 0.0245	+1.66 3.7 -1.26
3 Evap TFSW	0	0	0	0	0	0
4 TFSW	0	0	0	0	0	0



	treatment	female	% count	Input Column
1	1	1	.88	
2	1	1	.92	
3	1	1	.90	
4	1	2	.76	
5	1	2	.84	
6	1	2	.80	
7	1	3	1.00	
8	1	3	1.00	
9	1	3	.98	
10	1	4	.98	
11	1	4	.98	
12	1	4	1.00	
13	1	5	.40	
14	1	5	.40	
15	1	5	.48	
16	1	6	.44	
17	1	6	.52	
18	1	6	.34	
19	2	1	.22	
20	2	1	.20	
21	2	1	.16	
22	2	2	.12	
23	2	2	.10	
24	2	2	.08	
25	2	3	.04	
26	2	3	.04	
27	2	3	.02	
28	2	4	0	
29	2	4	0	
30	2	4	.02	
31	2	5	.02	
32	2	5	.02	
33	2	5	.04	
34	2	6	0	
35	2	6	0	
36	2	6	0	
37	3	1	0	
38	3	1	0	
39	3	1	0	
40	3	2	0	
41	3	2	0	
42	3	2	0	
43	3	3	0	
44	3	3	0	
45	3	3	0	
46	3	4	0	
47	3	4	0	
48	3	4	0	
49	3	5	0	
50	3	5	0	
51	3	5	0	
52	3	6	0	
53	3	6	0	
54	3	6	0	
55	4	1	0	
56	4	1	0	
57	4	1	0	
58	4	2	0	

	treatment	female	% count	Input Column
59	4	2	0.00	
60	4	2	0.00	
61	4	3	0.00	
62	4	3	0.00	
63	4	3	0.00	
64	4	4	0.00	
65	4	4	0.00	
66	4	4	0.00	
67	4	5	0.00	
68	4	5	0.00	
69	4	5	0.00	
70	4	6	0.00	
71	4	6	0.00	
72	4	6	0.00	

Data for Figure 5.4. Assessment of CMF Activity after Filtration through a 10 kilodalton filter

Treatment	Mean Percent Matured	Arcsine Mean Percent Matured	s.e. of Arcsine Mean Percent	Arcsine Mean Percent Matured +/- s.e.m	Back Transformed Values	Back Transformed Mean Percent Matured +/- s.e
1 CF	96.36	79.025	4.464	83.489 79.025 74.561	0.9872 0.9636 0.9295	+2.36 96.36 -3.41
2 CF Filtrate	0.82	5.227	1.799	7.026 5.227 3.428	0.0149 0.0082 0.0035	+0.67 0.82 -0.47
3 CF Wash	35.88	36.839	3.666	40.505 36.839 33.173	0.4218 0.3588 0.2998	+6.3 35.88 -5.90
4 TFSW	0.62	4.494	1.594	6.088 4.494 2.90 0.62	0.0113 0.0062 0.0026	+0.52 0.62 -0.36

	treatment	female	% count	Input Column
1	1	1	1.00	
2	1	1	1.00	
3	1	1	1.00	
4	1	2	.98	
5	1	2	.98	
6	1	2	1.00	
7	1	3	.96	
8	1	3	.98	
9	1	3	1.00	
10	1	4	1.00	
11	1	4	1.00	
12	1	4	.98	
13	1	5	1.00	
14	1	5	1.00	
15	1	5	1.00	
16	1	6	.38	
17	1	6	.36	
18	1	6	.44	
19	2	1	.08	
20	2	1	.08	
21	2	1	.06	
22	2	2	0	
23	2	2	0	
24	2	2	0	
25	2	3	0	
26	2	3	0	
27	2	3	0	
28	2	4	0	
29	2	4	0	
30	2	4	0	
31	2	5	0	
32	2	5	0	
33	2	5	0	
34	2	6	.08	
35	2	6	.08	
36	2	6	.06	
37	3	1	.12	
38	3	1	.14	
39	3	1	.16	
40	3	2	.38	
41	3	2	.40	
42	3	2	.42	
43	3	3	.48	
44	3	3	.54	
45	3	3	.60	
46	3	4	.22	
47	3	4	.26	
48	3	4	.30	
49	3	5	.80	
50	3	5	.70	
51	3	5	.84	
52	3	6	.06	
53	3	6	.10	
54	3	6	.20	
55	4	1	.08	
56	4	1	.06	
57	4	1	.08	
58	4	2	0	

	treatment	female	% count	Input Column
59	4	2	0.00	
60	4	2	0.00	
61	4	3	0.00	
62	4	3	0.00	
63	4	3	0.00	
64	4	4	0.00	
65	4	4	0.00	
66	4	4	0.00	
67	4	5	0.00	
68	4	5	0.00	
69	4	5	0.00	
70	4	6	.06	
71	4	6	.04	
72	4	6	.02	

Data for Figure 5.5. Assessment of the Effects of Tricyclic Anti-Depressants (TCAs) on the Maturational Ability of Oocytes

Treatment	Mean Percent Matured	Arcsine Mean Percent Matured	s.e. of Arcsine Mean Percent	Arcsine Mean Percent Matured +/- s.e.m	Back Transformed Values	Back Transformed Mean Percent Matured +/- s.e
1 TFSW	0	0	0	0	0	0
2 CMF	63.78	53.031	7.24	60.271 53.031 45.791	0.7545 0.6378 0.5140	+11.67 63.78 -12.38
3 ethanol	76.64	61.084	7.149	68.233 61.084 53.935	0.8621 0.7664 0.6528	+9.57 76.64 -11.36
4 Clomipramine 10 <sup>-4</sup>	80.23	63.58	7.199	70.757 63.558 56.359	0.8918 0.8023 0.6938	+8.95 80.23 -10.85
5 Clomipramine 10 <sup>-5</sup>	80.09	63.530	7.203	70.723 63.530 56.327	0.8908 0.8009 0.6921	+8.99 80.09 -10.88
6 Clomipramine 10 <sup>-6</sup>	84.35	66.733	7.291	74.024 66.733 59.442	0.9240 0.8435 0.7409	+8.05 84.35 -10.26
7 Desipramine 10 <sup>-4</sup>	81.74	64.714	7.542	72.256 64.714 57.172	0.9076 0.8174 0.7066	+9.02 81.74 -11.08
8 Desipramine 10 <sup>-5</sup>	82.80	65.541	7.252	72.793 65.541 58.289	0.9126 0.8280 0.7239	+8.46 82.80 -10.41
9 Desipramine 10 <sup>-6</sup>	79.95	63.351	6.911	70.262 63.351 56.44	0.8864 0.7995 0.6938	+8.69 79.95 -10.57
10 Imipramine 10 <sup>-4</sup>	74.55	59.730	6.680	66.410 59.730 53.051	0.8397 0.7455 0.6395	+9.42 74.55 -10.6
11 Imipramine 10 <sup>-5</sup>	70.66	57.207	6.431	63.638 57.207 50.776	0.8023 0.7066 0.6005	+9.57 70.66 -10.61
12 Imipramine 10 <sup>-6</sup>	76.35	60.858	7.254	68.112 60.858 53.604	0.8609 0.7635 0.6479	+9.74 76.35 -11.56



	treatment	female	% count	Input Column
1	1	1	0.00	
2	1	1	0.00	
3	1	1	0.00	
4	2	1	.98	
5	2	1	1.00	
6	2	1	1.00	
7	2	2	.90	
8	2	2	.92	
9	2	2	.90	
10	2	3	.80	
11	2	3	.76	
12	2	3	.70	
13	2	4	.26	
14	2	4	.28	
15	2	4	.20	
16	2	5	.90	
17	2	5	.92	
18	2	5	.90	
19	2	6	0.00	
20	2	6	0	
21	2	6	0	
22	3	1	.98	
23	3	1	.98	
24	3	1	1.00	
25	3	2	.94	
26	3	2	.96	
27	3	2	1.00	
28	3	3	.94	
29	3	3	.96	
30	3	3	.94	
31	3	4	.60	
32	3	4	.60	
33	3	4	.64	
34	3	5	.90	
35	3	5	.90	
36	3	5	.92	
37	3	6	0	
38	3	6	0	
39	3	6	0	
40	4	1	.96	
41	4	1	.94	
42	4	1	.98	
43	4	2	.98	
44	4	2	1.00	
45	4	2	1.00	
46	4	3	.96	
47	4	3	.96	
48	4	3	.98	
49	4	4	.74	
50	4	4	.76	
51	4	4	.74	
52	4	5	.92	
53	4	5	.94	
54	4	5	.96	
55	4	6	0	
56	4	6	0	
57	4	6	0	
58	5	1	1.00	

	treatment	female	% count	Input Column
59	5	1	1.00	
60	5	1	.98	
61	5	2	1.00	
62	5	2	.96	
63	5	2	.94	
64	5	3	.94	
65	5	3	.94	
66	5	3	.92	
67	5	4	.76	
68	5	4	.74	
69	5	4	.78	
70	5	5	.94	
71	5	5	.96	
72	5	5	.94	
73	5	6	0.00	
74	5	6	0.00	
75	5	6	0.00	
76	6	1	1.00	
77	6	1	1.00	
78	6	1	1.00	
79	6	2	.98	
80	6	2	1.00	
81	6	2	1.00	
82	6	3	.94	
83	6	3	.96	
84	6	3	1.00	
85	6	4	.80	
86	6	4	.84	
87	6	4	.80	
88	6	5	.92	
89	6	5	.90	
90	6	5	.96	
91	6	6	.02	
92	6	6	0	
93	6	6	0	
94	7	1	.98	
95	7	1	1.00	
96	7	1	1.00	
97	7	2	1.00	
98	7	2	1.00	
99	7	2	.96	
100	7	3	.80	
101	7	3	.98	
102	7	3	1.00	
103	7	4	.72	
104	7	4	.70	
105	7	4	.68	
106	7	5	.96	
107	7	5	.98	
108	7	5	.96	
109	7	6	0	
110	7	6	0	
111	7	6	0	
112	8	1	.96	
113	8	1	1.00	
114	8	1	.96	
115	8	2	.98	
116	8	2	1.00	

	treatment	female	% count	Input Column
117	8	2	1.00	
118	8	3	.94	
119	8	3	.94	
120	8	3	.98	
121	8	4	.90	
122	8	4	.92	
123	8	4	.92	
124	8	5	.92	
125	8	5	.90	
126	8	5	.92	
127	8	6	0.00	
128	8	6	0.00	
129	8	6	0.00	
130	9	1	.96	
131	9	1	.96	
132	9	1	.98	
133	9	2	.96	
134	9	2	.94	
135	9	2	.96	
136	9	3	.94	
137	9	3	.92	
138	9	3	.96	
139	9	4	.92	
140	9	4	.94	
141	9	4	.88	
142	9	5	.92	
143	9	5	.96	
144	9	5	.88	
145	9	6	0	
146	9	6	0	
147	9	6	0	
148	10	1	.94	
149	10	1	.94	
150	10	1	.92	
151	10	2	.92	
152	10	2	.94	
153	10	2	.92	
154	10	3	.94	
155	10	3	.94	
156	10	3	.94	
157	10	4	.70	
158	10	4	.70	
159	10	4	.72	
160	10	5	.94	
161	10	5	.92	
162	10	5	.96	
163	10	6	0	
164	10	6	0	
165	10	6	0	
166	11	1	.96	
167	11	1	.94	
168	11	1	.96	
169	11	2	.84	
170	11	2	.88	
171	11	2	.84	
172	11	3	.96	
173	11	3	.94	
174	11	3	.92	



	treatment	female	% count	Input Column
175	11	4	.70	
176	11	4	.72	
177	11	4	.70	
178	11	5	.80	
179	11	5	.86	
180	11	5	.80	
181	11	6	0.00	
182	11	6	0.00	
183	11	6	0.00	
184	12	1	.98	
185	12	1	1.00	
186	12	1	.98	
187	12	2	.96	
188	12	2	.94	
189	12	2	.92	
190	12	3	.96	
191	12	3	.94	
192	12	3	1.00	
193	12	4	.66	
194	12	4	.56	
195	12	4	.44	
196	12	5	.94	
197	12	5	.94	
198	12	5	.92	
199	12	6	0	
200	12	6	0	
201	12	6	0	